BAD BREATH

A MULTIDISCIPLINARY APPROACH

DANIEL van STEENBERGHE & MEL ROSENBERG
EDITORS

BAD BREATH

BAD BREATH

A Multidisciplinary Approach

DANIEL van STEENBERGHE and MEL ROSENBERG

Editors

Leuven University Press
1996

ISBN 90 6186 779 7

D/1996/1869/61

Cover : W. Platteborze

Contents

Microbiology and breath odour

Clinical Assessment and Detection of Breath Odour

Treatment of Breath Odour

Consensus Reports

Preface

M. Rosenberg

According to a tongue in cheek article carried by "Reuters" and published in the Jerusalem Post on December 26th, 1995, Men's Health chose the "Second International Workshop on Oral Malodour" as "the worst conference to attend in 1995". But to 140 scientists from 16 countries around the world, it was quite the opposite. I still have trouble believing that this area of research which was generally considered to be "off the wall" only a few years ago has gained so much momentum.

If one goes back over the literature, it becomes clear that during any given period, only one or two groups of scientists were willing to devote themselves to studying this problem with any tenacity. In many cases, researchers made a sole, oftentimes important, contribution to the field (Grapp, for example) and then moved on to other pursuits. In this context, the monumental contribution of Joseph Tonzetich to this field is even more pronounced. Dr. Tonzetich, for more than thirty years, has focused his energy, ingenuity and acumen on this subject. Dr. Tonzetich has also raised an international cadre of scientists who have remained interested in volatile malodorous oral compounds and their effect on our health.

To some extent, the burgeoning current interest in bad breath research is fueled by economic considerations. Although "breath freshening" has always been big business, the active entry of dentists into this area over the past four years has created a financial stir. Indeed, there are hundreds of millions of dollars to be made diagnosing and treating the large segment of the population (perhaps 25 million in the US alone) who either suffer from bad breath, or assume that they do. Thus far, the business is way ahead of the science. Dentists pay large sums of money to participate in courses that teach them, almost instantly, to be so-called breath experts. In many instances, the emphasis of such courses is on the fiscal health of the dentists, rather than the physical health of the patients. As a result, patients pay large sums of money to receive a sometimes dubious diagnosis, and a treatment regimen based on a brand of mouthwash which the dentist buys and sells.

As scientists, we are now playing catch up, trying to bring a balanced scientific background to the thousands of dentists already involved in this area.

Hopefully many of them will buy (and read) this book. Bad breath: a multidisciplinary approach contains the latest thoughts and experimental data of scientists from around the world who are involved in this area.

But even if we fail to appreciably influence the "fresh breath" dentists currently in the field, we must keep our eyes on the future. Dental and medical schools must teach more about breath odour diagnosis and treatment at the undergraduate and graduate levels. Curricula should incorporate both "classic" and recent scientific findings.

The reader looking for all the absolute answers on bad breath is bound to be a little disappointed by the present volume. Bad breath: a multidisciplinary approach does not set out guidelines on the best way to measure or treat bad breath. As with other complex issues (periodontitis comes immediately to mind), the more we study, the clearer our lack of knowledge becomes. As with the previous book "Bad breath: research perspectives", different authors have expressed divergent viewpoints on various themes (for example the nature of the gases involved in various breath odours, the types of bacteria responsible) and have proposed different treatment regimens. Nevertheless, the participants at the Workshop developed consensus papers, published here, which deal with the central issues at hand:

1. What criteria are currently important in measurement and diagnosis of breath odours in the clinic and laboratory?
2. How should the effectiveness of mouthrinses, toothpastes and other treatments be compared in scientific studies?
3. What areas should be focused on the in future research?

In closing, I would like to express my appreciation to Daniel van Steenberghe. Without his keen interest and endeavour, the First International Workshop would have remained a solitary isolated event. This book, the planned establishment of a society for breath odour research, and the continued enthusiasm towards further meetings and research are largely to his credit.

Introduction

D. van Steenberghe

Even with the many channels presently available for the transfer of knowledge, a gap often exists between scientific innovations and clinical practice. When it comes to breath odour research lack of interest remained for some two decades between the pioneering work in the sixties of Tonzetich and co-workers and the rest of the international research community even from disciplines everyone would expect to be involved: ENT, periodontology, internal medicine, pharmacology, microbiology ... It was only in 1992 that the Journal of Periodontology published a series of articles on the subject based on an IADR satellite symposium organised by Mel Rosenberg. He also organised in 1993 the First International Workshop on Oral Malodour in Israel. This meeting acted as an eye-opener for many. Although only some 60 people from six countries attended, they represented a large variety of disciplines ranging from psychology to chemistry, from biochemistry to microbiology, from periodontology to oto-rhino-laryngology.

Representatives from major companies involved in the development of oral hygiene products understood the importance of the event. It also made me realise how inattentive we had been for so long also in a department, like ours, to the frequent problem of breath odour, a term which does not possess the negative connotation of oral malodour. Encouraged by the initiative of L. Feenstra, head of our ENT department to start a multidisciplinary outpatient clinic in our university hospital, and approached by Mel Rosenberg to repeat the Israeli Workshop format, the Second International Workshop on Oral Malodour in Leuven was organised for October 1995.

This time no less than 140 (several other applicants were refused) researchers and clinicians from 16 countries gathered for two full days in the romantic atmosphere of the 17th Century béguinage, as a village in the town. Short walks over the old cobble stoned streets from the conference house to the hotel or the Faculty Club took place in an "Indian summer" climate.
This meeting became successful thanks to the close collaboration of the organising committee members (Louw Feenstra, ENT, Catholic University Leuven, Belgium; Israel Kleinberg, Oral Biology, State University, New York, USA; Walter Loesche, Microbiology, The University of Michigan, USA; Hideo Miyazaki, Preventive Dentistry, Kyushu Dental College, Kitakyushu, Japan;

Michael Newman, Periodontology, UCLA, Los Angeles, USA, Mel Rosenberg, Microbiology, Tel-Aviv University, Israel; Joseph Tonzetich, Oral Biology, University of British Columbia, Canada). The active participation of the attendees was impressive. Most precious was the entrepreneurial spirit of Mrs. Miranda Marechal, who headed the secretarial organisation. The help of members of the department of periodontology in Leuven is acknowledged. The workshop was generously supported by two golden (***Teledyne Water Pik, Zendium***) and six silver sponsors (*Colgate Palmolive, Knowell Technology, Lion Corporation, Sunstar Inc., Unilever Research, Warner Lambert*).

Besides the oral and poster presentations, the vast majority of which are reported in this book in an edited version, there were three working groups who defined consensus statements on the following themes :

- The clinical approach of breath odour
- Toothpastes and mouthwashes to control breath odour
- Value of commercially available breath odour meters

Their conclusions are to be found at the end of this book (p. 285).

The meeting was characterised by lively discussions where cross-fertilisation between different disciplines appeared fruitful. Concern was voiced regarding commercial "breath freshening mouthrinses" lacking scientific credentials, as well as so-called "fresh breath" clinics, offering diagnosis based solely on sulphide monitoring.

This meeting also led to the establishment of a scientific society "The International Society for Breath Odour Research" with the abovementioned organising committee members as an interim board and a secretariat in the hands of Prof. Mel Rosenberg, Section of Oral Biology; The Maurice and Gabriela Goldschleger School of Dental Medicine, Tel-Aviv University, Ramat-Aviv, Israel, Tel 972.3.642.93.11, Fax 972.3.640.9250 or 972.3.640.6023, email: melros@ccsg.tau.ac.il.

Plans have been forged to perpetuate the workshop every two years under the format of an international symposium.

Periodontal Parameters and Volatile Sulphur Compounds

The Role of Periodontitis in Oral Malodour: Clinical Perspectives

*M. G. Newman**

In this article the term *oral malodour* will be used to describe a foul or offensive odour emanating from the oral cavity. For this discussion, the intraoral environment consists of all of the structures in the oral cavity including the tongue, the tonsils, the soft palate and the oropharynx. The purpose of this review is to help the clinician formulate a model of how periodontal infections relate to the diagnosis and management of patients seeking treatment for oral malodour.

Bad breath is of major importance socially and economically. It may be caused by a number of factors and can occasionally be linked to more serious underlying medical etiologies (Fig. 1). In most cases, bad breath originates within the oral cavity. Examples of other sources include: the nasal passages, alcohol ingestion, uremia associated with kidney dysfunction and metabolic disorders. (1,2) Although these may be potentially important signs, they will not be discussed here.

Malodour caused by smoking and food intake, such as garlic, onions, and other foods is a distinctly different problem and is not the subject of this report. A large number of products have recently been promoted to reduce these odours which are called "bad breath" in commercial advertisements.

Commercial product development has concentrated primarily on masking or blocking the offensive odours through the use of mouthrinses, chewing gum and candy-like lozenges or pastilles. Some products claim to reduce bad breath by killing "millions" of bacteria in the oral cavity and others claim to eliminate or reduce odours caused by food intake and digestion. Conventional and adjunctive oral hygiene methods are important because the directly remove plaque and food debris from the teeth, subgingival areas, the tongue and other parts of the oral cavity.

Unsubstantiated commercial advertisements in the United States lay press claim as many as 25 million Americans "suffer" from bad breath. The exact numbers or percentage of the population who have oral malodour is very difficult to determine because there are no universally accepted criteria of diagnosis. For example there is not yet an accepted "cut-off 'line for the level

---

* M.G. Newman, School of Dentistry, Section of Periodontics, UCLA, 10833 Le Conte Avenue, Los Angeles, CA 90024-1668, USA.

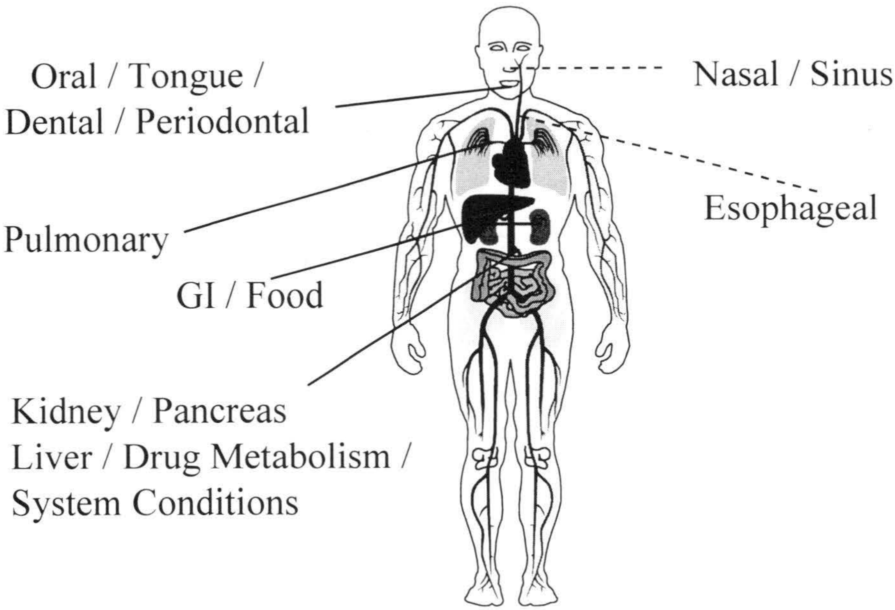


Fig. 1: Sources of oral malodour can originate in one or several sites within the body. Some odours may be of significant medical diagnostic importance.

of oral volatile sulphur compounds (VSC's) which places a patient into the malodour category. At 75 ppb VSC concentration, the level at which is suggested to be meaningful, Miyazaki et al (3) determined that up to 25% of the Japanese population would have oral malodour.

Periodontitis - sufficient but not necessary

The vast majority of patients who seek treatment for oral malodour in the dentists' or periodontologists' office have an intraoral etiology. However as Rosenberg (4) points out, "the relationship between oral malodour and periodontal diseases is a perplexing one". Many patients with a chief complaint of oral malodour have some level of gingival and or periodontal pathology ***sufficient*** to be the etiology, *but clearly periodontal pathology is not a prerequisite or is it* ***necessary*** *for the production of oral malodour.* This periodontitis paradigm is a logical extension of the recognition that putrefaction of food debris and degradation of proteins in the periodontal pocket occurs from the action of the oral microbiota and in particular the microorganisms associated with periodontitis. (5) Associations of very poor

(or non-existent) oral hygiene and necrotizing oral lesions such as acute necrotizing ulcerative gingivitis in people with oral malodour, have perpetuated the idea that there is always a periodontal etiology.

Examination of the status of the patient's restorative dentistry in a individual who complains of oral malodour may reveal one or a number of the following factors which can be prime "suspects": large carious lesions, exposed necrotic pulp tissue, open margins, sites of food impaction, tight embrasures, washed out cement, abraded or eroded restorations, and, retentive clasps, bars and attachments. All of these findings can contribute to the production of malodour because they can retain bacteria and debris. Systematic repair and/or replacement often improves the chances of success when the etiology is a dental problem. From a practical view, it is often necessary to prioritize and sequence the treatment since time and expense do not permit patients to get all their work done at once.

Periodontal associations

Commonly held clinical opinion suggests the following (greatly simplified) tenets about how periodontitis might contribute to the development and production of oral malodour:

1. Periodontitis is caused by anaerobic and facultative protein utilizing bacteria.
2. These bacteria are located in periodontal pockets and older established plaque where sulphur containing substrates are available.
3. Subgingival anaerobes, such as *Porphyromonas gingivalis, Prevotella* spp., and many others, reduce sulphur containing amino acids to hydrogen sulphide H_2S, methyl mercaptan, CH_3SH and dimethyl sulphide CH_3SCH_3 referred to as VSC (volatile sulphur compounds). Volatile sulphur compounds are some of the specific by-products of bacterial metabolism of many different host substrates including but not limited to: crevicular fluid constituents, leucocytes, gingival bleeding, epithelial cells, other bacteria and their constituents, (6) (Table 1).
4. These VSC are released into the oral environment where they mix with expired air and contribute to malodour.
5. VSC might contribute to the pathogenesis of periodontitis because they have pathogenic potential on a variety of host cells and processes.
6. The plaque associated with periodontitis lesions lead bacteria to other oral sites such as the tongue dorsum where they colonize and contribute to the total oral malodour status (Fig. 2).

Table 1: Microbiologic targets for anti-malodour treatment.

Target ⇒ Reduction or elimination of specific bacteria capable of producing VSC's
Chronic Adult Periodontitis (CAP)
Porphyromonas gingivalis
Prevotella intermedia/nigrescens
Actinobacillus actinomycetemcomitans (Aa)
Campylobacter rectus
Fusobacterium species
Peptostreptococcus micros
Bacteroides forsythus
Eubacterium species
Treponema species
Refractory Adult Periodontitis
CAP + Various exogenous species:
Pseudomonas species
Enteric rods
Candida species
Miscellaneous other microorganisms frequently encountered
Acute Necrotizing Periodontitis
Same as Refractory Adult Periodontitis + Spirochetes
Acute Necrotizing Ulcerative Gingivitis
Same as Acute Necrotizing Periodontitis
Tongue and Tonsil Flora
Same as CAP **+** *Capnocytophaga* sp. and *Rothia* sp.

Many patients who have periodontitis do not complain about oral malodour even though these individuals often have higher levels of VSC. Many other oral malodour etiologic factors can be found in patients with periodontitis, for example a low salivary flow rate and the quantity of tongue coating they have. Yaegaki and Sanada (7) found more wet weight of tongue coating in patients with periodontitis but they did not determine whether the coating contained higher numbers of odour causing bacteria. Indeed, Bosy et al (8) suggests that periodontitis alone may not be associated with oral malodour.

In periodontally stable, edentulous or healthy individuals with no detectable dental cause, oral malodour production may be associated with degradation of proteins, sulphur containing organic compounds on the surface of the tongue, tonsils and in the saliva by bacteria and host enzymes. Many patients with a healthy oral status have oral malodour caused by expired air passing over sulphur and protein rich mucous secretions associated with post-nasal drip (4).

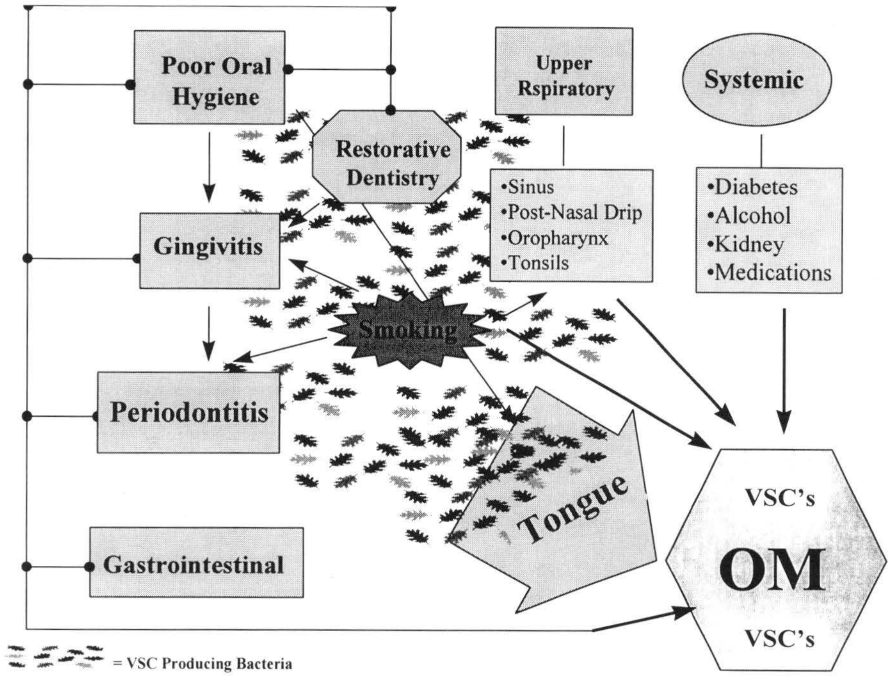


Fig. 2: Etiological factors associated with oral malodour: this is often a combination of different intraoral, systemic and external factors. Often VSC producing bacteria from gingival and periodontal inflammation, poor oral hygiene and the upper respiratory tract have the potential to play a primary and/or a secondary role in producing malodour. The tongue can be "seeded" by bacteria from many different sources.

Endosseous implants and oral malodour

There has been virtually nothing written about oral malodour in patients with endosseous implants, yet there is a constant stream of anecdotal information that peri-implant plaque and inflammation might contribute to malodour complaints. From a theoretical view, many of the same principles associated with periodontitis apply to periimplantitis (9). Similar microbiota, anatomical "hiding" places for bacteria, inaccessible areas for oral hygiene, typically deeper peri-implant gingival tissues and other considerations suggest that these areas have the potential to contribute, on an individual patient basis, to oral malodour production. Patients with implant supported oral and maxillofacial prostheses also have malodour. In these cases strict oral hygiene, topical agents including fluorides, chlorhexidine, artificial saliva and systemic medications are needed.

It seems that a prudent course of action in these patients is to encourage the strictest levels of oral hygiene that the patient can perform. In these patients adjunctive oral hygiene measures such as irrigation, mechanical toothbrushes and antimicrobial solutions can materially enhance the desired clinical outcomes. (10, 11)

Gingival associations

Oral malodour associated with gingival problems appears to be due to the bacterial degradation of odour causing substrates. The bacterial composition of plaque which is allowed to build-up due to inadequate oral hygiene contains larger numbers of Gram-negative bacteria which utilize proteins from a variety of sources as nutrients. As a consequence of metabolism, volatile compounds are produced and released into the environment during normal respiration and talking. When plaque is retained for a period of time, gingivitis develops and further contributes to the oral malodour cycle. Inflammation and swelling contribute to the process by furnishing host proteins and amino acids through the production of crevicular fluid and the swelling of the gingiva increases the volume of the pocket which in turn increases the amount of odour causing bacteria which are retained. As in the case of periodontitis, gingivitis is not necessarily the cause of oral malodour. When it is present it is one of the causative factors which should be treated. (Fig. 2)

In relatively few circumstances, gingival pathology associated with necrosis, granulomatous proliferation or ulceration, may cause malodour. Acute gingival problems such as acute herpetic gingivostomatitis, persistent gingival bleeding associated with blood dyscrasias, gingival fibromatosis and other conditions may contribute to the overall malodour status. All of these conditions require definitive diagnosis and appropriate treatment. There are also situations such as gingival tissue flaps connected with third molar eruption and healing alveolar sockets associated with tooth extraction which can cause malodour. Environmental factors such as smoking exacerbate plaque retention and decrease gingival metabolism. Both factors reduce the oxygen tension in the local environment which favours growth of anaerobic protein-utilizing bacteria.

Diagnosis

There are several ways in which patients are diagnosed with oral malodour and enter the formal treatment phase. They may seek a "remedy" directly from the dentist's or periodontologist's office or clinic, they may be referred by another health professional, or the patient could be diagnosed in the dental office as part of routine oral health care. The actual formal diagnosis follows a sequence of subjective and objective procedures which may vary according to the original entry point into the health system. Whatever the starting point, the diagnosis phase must be comprehensive and complete.

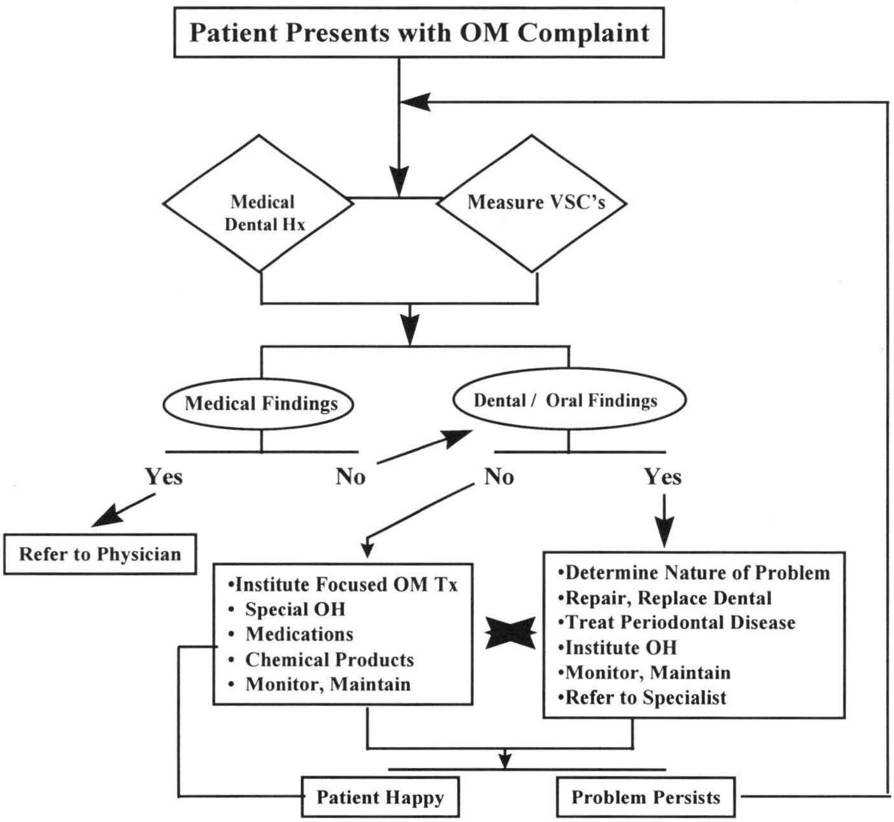


Fig. 3: Oral malodour patient management algorithm.

The diagnosis and treatment algorithm

An algorithm is a step-by-step problem-solving procedure, especially an established procedure for solving a problem in a finite number of steps. Figure 3 depicts a general oral malodour algorithm which can be used in a variety of clinical settings. Once the patient presents for diagnosis, a complete medical and oral care history must be taken and evaluated. If the patient has a finding which suggests a medical etiology the patient must be referred to a physician. Most Oral Malodour Clinics or private offices with experience treating these patients have developed a multidisciplinary approach which provides for medical consultation. Often this will include an Ear Nose and Throat specialist.

Patients who do not appear to have a medical problem are referred to the dentist or periodontologist. There are no standard and accepted protocols for the treatment of oral malodour even though many clinicians claim to be successful. However, all protocols contain the basic elements and include standard dental and periodontal treatment *and* focused oral malodour treatments. If the patient does not respond, the diagnosis cycle must be restarted to look for additional factors not evaluated or discovered during the initial examination. Both patient and health care professionals be aware and be careful of "quick fixes" and schemes that avoid giving the patient incomplete treatment, for example not treating obvious periodontal problems when they exist, but instead giving the patient a mouthrinse treatment to reduce the malodour.

Measuring VSC as part of diagnosis and treatment is essential because it provides an objective means of determining the levels of the products associated with malodour. Rosenberg et al (12) and others have demonstrated reasonable reproducibility of these measurements and they appear to be well correlated with organoleptic assessments. However there are also limitations such as lack of specificity and universal calibration.

The result of professional and patient intervention must be a satisfied and happy patient judged according to the patient's standards and expectations.

Matching oral hygiene technology with the patient's needs

Any treatment approach directed at eliminating or controlling oral malodour will include various combinations of oral hygiene procedures. Matching the level and intensity of oral hygiene precisely with the patients needs and preferences will maximize treatment outcomes. (Fig. 4).

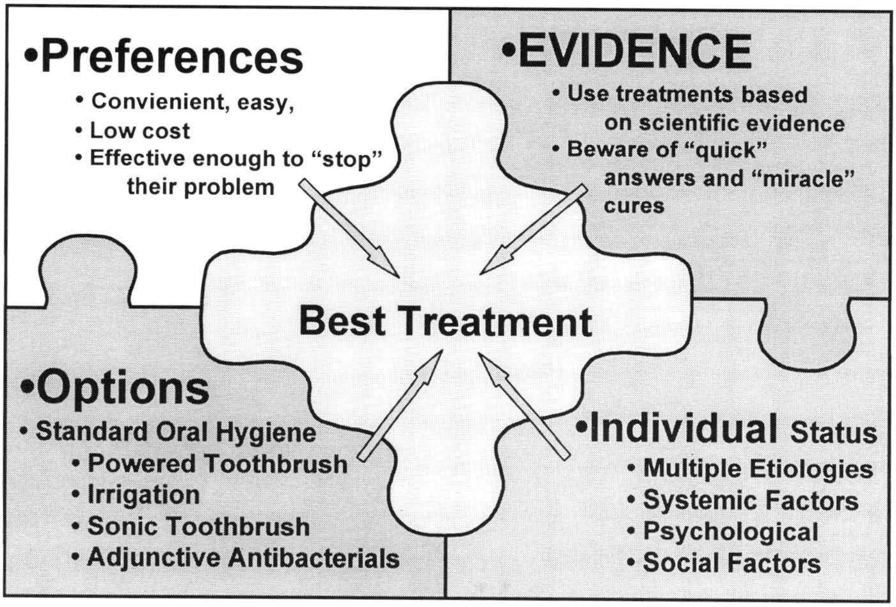


Fig. 4: Matching the patient's individual needs and preferences with oral hygiene technology options is the key to compliance and therapeutic success.

For patients with relatively healthy periodontal status, good motivation and manual dexterity, normal brushing and flossing are probably adequate. For patients with complicating factors such as fixed and/or removable prosthesis more "advanced" hygiene methods and devices should be employed. These include electric toothbrushes, oral irrigation with and without antimicrobial agents (13, 14) and sonic and ultrasonic toothbrushes.

Earlier studies by Temple et al (15) have demonstrated the ability of oral irrigation with water to remove the vast majority of soluble and insoluble particulate matter from the oral cavity compared to rinsing. (Fig. 5) Although not tested directly, this method may have the potential to greatly remove substrate from a variety of intraoral sites. Eakle et al (16) have demonstrated the ability of oral irrigation to penetrate deep into periodontal pockets and gingival sulci to remove plaque bacteria.

Many geriatric patients and other individuals who have impaired abilities to clean their teeth can benefit from the electric toothbrushes and whenever possible from irrigation and rinsing. As with any therapy the risk versus the benefit must be determined. In the case of rinsing with alcohol containing products , clinicians must weigh the benefit of the active ingredient versus the risk of high alcohol content.

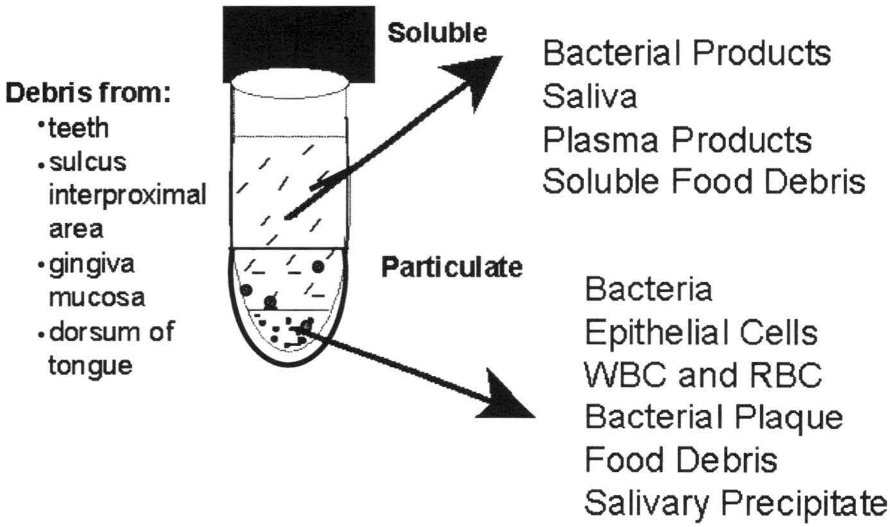


Fig. 5: Particulate and soluble debris removed by oral hygiene which included the use of oral irrigation. Oral hygiene which included rinsing does not remove as much material. (Adapted from reference 15).

In many other patients, where their level of conventional oral hygiene is adequate but their oral malodour problem persists, it is important to integrate tongue cleaning into their daily regimen. Besides the classical toothbrush, various devices have been discussed and many of them appear to work as long as the posterior parts of the tongue are cleaned.

Combining mechanical methods with effective antibacterial products can be confusing since there are so many brands and products on the market. Only products which are supported by scientifically valid evidence (17) for both safety *and efficacy* can be recommended. Antibacterial products such as triclosan, stannous fluoride, chlorhexidine (11) and others must be monitored for their effectiveness on an individual patient basis. Together with normal periodontal maintenance various combinations of oral hygiene methods can be effective in reducing oral malodour in some patients. This includes removal and cleaning of prostheses. Unfortunately there are few if any randomized clinical trials which have provided the clinician with a sufficient level of evidence to allow better treatment decisions.

References

1. Carranza F.A. Jr. (1996) Clinical Diagnosis. In: Carranza F.A and Newman M.G. (Eds.). *Clinical Periodontology.* 8th Edition. WB Saunders. Philadelphia.
2. Preti G., Lawley H.J., Hormann C.A., Cowart B.J., Feldman R.S., Lowry L.D. and Young I-M. (1995) Non-Oral Aspects of Oral Malodour. In: Rosenberg M. (Ed). *Bad Breath: Research Perspectives.* Ramot Publishing. Tel Aviv University, pp149-173.
3. Miyazaki H., Sakao S., Katoh Y. and Takehara T. (1995) Correlation between volatile sulfur compounds and certain oral health measurements in the general population. *J Periodontol* 66:679-684.
4. Rosenberg M. (1995) Introduction. In: Rosenberg M. (Ed) *Bad Breath: Research Perspectives.* Ramot Publishing. Tel Aviv University, pp 1-12.
5. De Boever E.H., De Uzeda M. and Loesche W.J. (1994) Relationship between volatile sulfur compounds, BANA-hydrolyzing bacteria and gingival health in patients with and without complaints of oral malodour. *J Clin Dent* 4:114-119
6. Van Winkelhoff A.J., Van der Velden U., Winkel E.G., de Graaff J. (1986) Black pigmented *Bacteroides* and motile organisms on oral mucosal surfaces in individuals with and without periodontal breakdown. *J Periodont Res* 21:434-439.
7. Yaegaki K. and Sanada K. (1992) Volatile sulfur compounds in mouth air from clinically healthy subjects and patients with periodontal disease. *J Periodont Res* 27:233-238.
8. Bosy A., Kulkarni G.V., Rosenberg M. and McCulloch C.A.G. (1994) Relationship of oral malodour to periodontitis: Evidence of independence in discrete subpopulations. *J Periodontol* 65:37-46.
9. Newman M.G. and Marinho V.C. (1994) Assessing bacterial risk factors for periodontitis and perimplantitis: using evidence to enhance outcomes. *Compendium* 15:958-962.
10. Ciancio S.G., Lanciello F., Shibly O., Vitello M. and Mather M. (1995) Effect of an antiseptic mouthrinse on implant maintenance: plaque and periimplant gingival tissues. *J Periodontol* 66:962-965.
11. Veksler A., Kayrouz G.A. and Newman M.G. (1992) Reduction of salivary bacteria by pre-procedural rinses with chlorhexidine. *J Periodontol* 63:649-651.
12. Rosenberg M., Kulkarni G.V., Bosy A. and McCulloch C.A.G. (1991) Reproducibility and sensitivity of oral malodour measurements with a portable sulfide monitor. *J Dent Res* 70:1436-1440.
13. Newman M.G., Cattabriga M., Etienne, Kornman K.S., Doherty F.,

Moore D.J. and Ross C. (1994) Effectiveness of adjunctive irrigation in early periodontitis: multicenter evaluation. *J Periodontol* 65:224-229.
14. Fine J.B., Harper D.S., Gordon J.M., Hovliaras C.A. and Charles C.H. (1994) Short term microbiological and clinical effects of subgingival irrigation with an antimicrobial rinse. *J Periodontol* 65:30-36.
15. Temple T.R., Marcil J.F.A. and Seibert J.S. (1975) Comparison of water irrigation and oral rinsing on clearance of soluble and particulate materials from the oral cavity. *J Periodontol* 46:301-306.
16. Eakle W.S., Ford C. and Boyd R.L. (1986) Depth of penetration in periodontal pockets with oral irrigation. *J Clin Periodontol* 13:39-44.
17. Newman M.G. and McGuire M.K. (1995) Evidence-based periodontal treatment 11. Predictable regeneration treatment. *Int J Periodont Rest Dent* 15:116-127.

Where Gastroenterology and Periodontology meet: Determination of Oral Volatile Organic Compounds using Closed-loop Trapping and High-resolution Gas Chromatography-ion Trap Detection

*D. Claus, B. Geypens, P. Rutgeerts, J. Ghyselen, K. Hoshi, D. van Steenberghe and Y. Ghoos**

A method is described for the analysis of volatile organic compounds in saliva and tongue coating samples. The technique is based on an off-line preconcentration by means of a closed-loop trapping system followed by high-resolution gas chromatography-ion trap detection (HRGC-ITD) for separation and identification of the compounds.
The technique has been validated for pattern recognition in saliva and tongue coating samples. It is considered a very promising tool in further research on oral malodour.

Introduction

For many years, volatile substances have been considered an important object of research in medicine. Organic volatiles are studied in different human matrices such as saliva (1, 2), tissue homogenates, cerebrospinal fluid, serum (3), breath (4, 5, 6), flatus (7, 8), faeces (9, 10, 11) and urine (11). In oral malodour, the detection and identification of volatile substances can help solve the malodorous problem.

The first aim of this study was to validate a technique, already used in faecal and urinary samples, for salivary and tongue coating samples.

The second aim was to demonstrate the formation of volatile bacterial fermentation substances when a protein substrate was added to different tongue coating samples.

---

* D. Claus*, B. Geypens*, P. Rutgeerts*, J. Ghyselen°, K. Hoshi°, D. van Steenberghe° and Y. Ghoos*, Department of Gastrointestinal Research* and Department of Periodontology°, Faculty of Medicine, Catholic University Leuven, Capucijnenvoer 7, 3000 Leuven, Belgium.

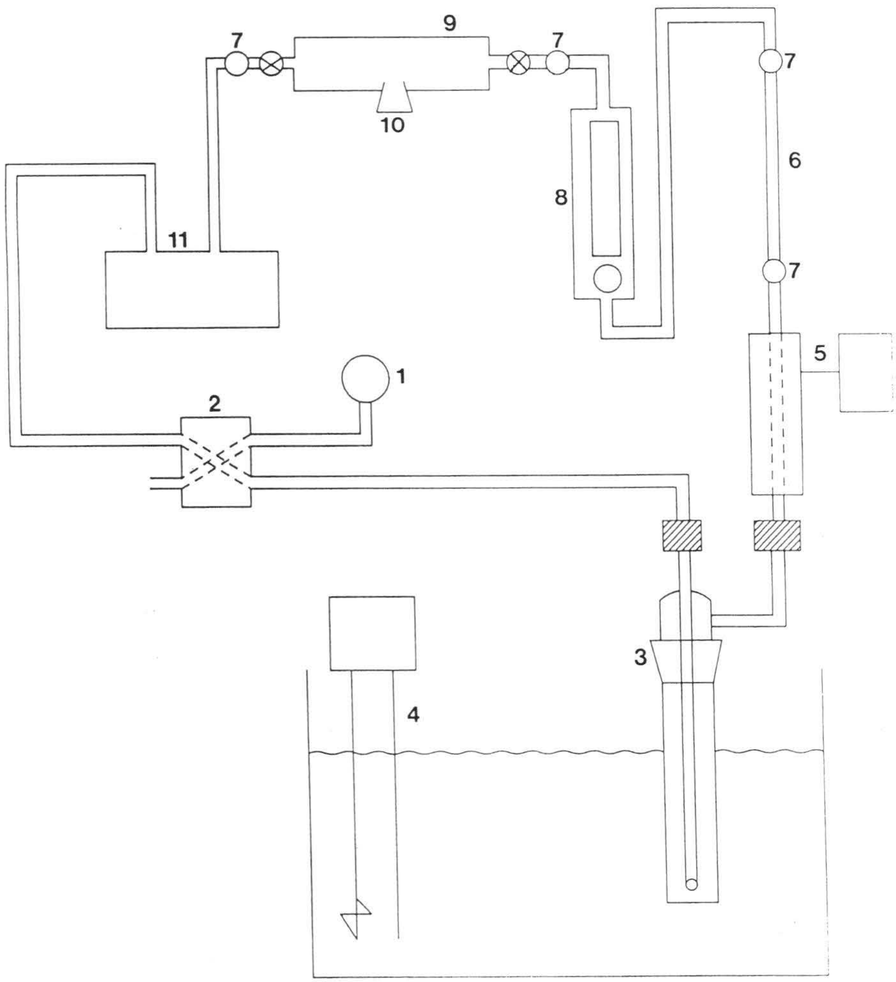


Fig. 1: The closed-loop trapping system: {1} nitrogen supply; {2} 4-way valve; {3} sample container; {4} thermostatic bath; {5} water condensing system; {6} adsorption trap; {7} Rotulex joints; {8} flow meter; {9} gas bulb; {10} septum; {11} pump system. J. Chromatogr. A;665;334. With permission of Elsevier Science.

Experimental

a. Closed-loop trapping apparatus

The device in this paper is identical to that one described by Ghoos and coworkers (13) (Fig. 1).

Numbers in brackets refer to the figure.

Pure nitrogen (99.9995%) (Air Products, Vilvoorde, Belgium) {1} enters the system by a 4-way valve (Whitey, Highland Heights, OH, USA) {2} with a stainless-steel inner housing. Switching the valve opens or closes the loop. When the valve is closed, the nitrogen gas flows continuously through the loop. When the valve is open, the system is continuously flushed by pure nitrogen. For a very clean background, a charcoal filter between the nitrogen gas bulb and the 4-way valve is recommended.

The gas enters the sample container {3} vertically and leaves through an elbow. This vessel is made of glass and is easily connectable with finger tight Cajon ultra-torr couplings (Macedonia, OH, USA). The sample vessel is dipped in a thermostated water bath {4} at 37°C. Just before the adsorption trap, a glass tube is passed through a heating block {5} to minimize possible condensation of water from the gas leaving the sample container. The temperature of the heated block is kept at 60°C above the temperature of the sample container.

The adsorption trap {6} is filled with 160 mg Tenax GR (Chrompack, Middelburg, Netherlands) and is connected in the system by Rotulex knee-joints {7}, providing excellent tightness and a rapid exchange. The traps are preconditioned overnight in an oven (SoVirel, France) at 240°C with a gentle stream of nitrogen (99.9995%). They are used the same day. An aluminium foil is kept around to prevent Tenax from degradation by light. The gas bulb (Alltech, Laarne, Belgium) {9} has a volume of 125 ml and is modified with Rotulex knee-joints at the ends for quick exchange. Through the septum {10} of the flask it is possible to add internal standards or to withdraw samples. A polytetrafluoroethylene (PTFE) membrane pump (KNF Neuberger, Freiburg, Germany) {11} and a flow meter (GEO/National, Hasbrouck Heights, NJ, USA) {8} provide the flow through the system. The pump has a stainless-steel diaphragm coated with PTFE. This diaphragm is easily accessible for cleaning or exchange. The outlet of the pump is connected to the 4-way valve. The flow through the system is about 250ml/min. All tubing is made of 1/8 in. (1 in. = 2.54 cm) stainless steel, except for the connection between the sample container and the adsorption trap, which is made of glass.

b. Desorption-separation-detection system

To release the Volatile Organic Compounds (VOCs) from the adsorption trap, a modified thermodesorption cold trap (TCT) injector is used (12, 13).

Thermodesorption and cryofocusing were carried out in the following manner: holding the desorption trap at 200 °C for 5 minutes with a helium (99.9995%) (Air Products) flow of 20 ml/min (sufficient for the desorption of the VOCs). Meanwhile the cryotrap, a 0.32 mm uncoated deactivated fused silica, was held at -120 °C. Breakthrough was non-existent under these conditions.

The VOCs concentrated in this manner in the cryotrap were released into the analytical column by heating the cryotrap to 200 °C for another 20 minutes.

Separation and identification of the compounds were performed on a HRGC-ITD system. The gas chromatograph was a Vega 6000 instrument (Carlo Erba, Milan, Italy) equipped with a cryostat (cryo 620) with carbon dioxide, a split-splitless injector and a TCT injector. The analytical column was a 25 m x 0.32 mm CP-Sil 5CB, film thickness 1.2 µm (Chrompack). Helium (99.9995%) at 70 kPa inlet pressure was the carrier gas. Oven temperature program: 30°C (5min); 5°C/min; 200°C (6 min). The analytical column was coupled to the ion trap detector , model ITD 700 (Finnigan, San José, CA, USA) by means of an open split interface. The transferline was held at 220°C.

Detection was carried out in electron impact mode by multiple ion monitoring from m/z 34-39, m/z 41-43 and m/z 45-300. Automatic Gain Control was "on". Scans were averaged every second, as the result of 5 µscans.

c. Reagents and standard solutions

Tenax GR was purchased from Chrompack (Middelburg, Netherlands).

Na_2HPO_4. $12H_2O$ and NaH_2PO_4. $2H_2O$ were purchased from Fluka (Buchs, Switzerland). Sterile pyrogene-free water was obtained from Baxter (Lessines, Belgium). The internal standard mixture containing bromo-chloromethane, 1,4-dichlorobutane and 1-chloro-2-bromopropane, (2 mg/ml each) was purchased from Supelco (Bellefonte, PA, USA).

For saliva and tongue coating analysis, 2.5 µl internal standard mixture was diluted in 1 ml sterile pyrogene-free water. An aliquot of 5 µl of this solution is used for standardisation, so an amount of 25 ng for each standard on the analytical column is achieved. The standard solution was prepared daily and kept at 4 °C in the dark.

The buffer solution contained 2.9 g $Na_2HPO_4.12H_2O$ and 0.7 g $NaH_2PO_4.2H_2O$ per litre (pH 7). This solution was freshly made every day with pyrogene-free water.

Casein was purchased from the Melkindustrie (Veghel, Netherlands).

d. Sample preparation

Saliva samples

One ml of fresh morning saliva was diluted with 2 ml of buffer solution in the sample container and directly plugged into the closed-loop trapping system.

Tongue coating samples

Between 30 and 40 mg of coating was taken from the dorso-posterior surface of the tongue and dissolved in 3 ml buffer solution in the sample container. The container was flushed with pure nitrogen and firmly closed by means of a clamp and two Swagelok® fittings with PTFE seals at the small glass ends. The sample container was placed in an incubator (Polylab, Antwerpen, Belgium) at 37 °C for 24 h before it was plugged into the closed-loop trapping system.

For fermentation pattern recognition, tongue coating samples were taken, diluted into 6 ml buffer solution and equally divided into 2 sample containers. To one container, 5 mg casein was added. After flushing with pure nitrogen, the sample containers were firmly closed and incubated for 24 h at 37 °C before sample analysis.

e. Methods

For determination of the samples, an adsorption trap was added to the closed-loop system just before the sample container was plugged in. The loop was closed by means of the 4-way valve. Internal standard solution 5 µl was added through the septum of the gas bulb. The sample container was immersed into the water bath at 37°C. After 15 minutes of closed-loop trapping, the adsorption trap was removed and closed on both ends with glass cocks and Rotulex clamps until analysis was carried out on the same day.

Internal standards could not be added to the samples before incubation because some bacteria may break down halogenated hydrocarbons (14).

Between each sample run a wash-out period of 30 minutes was performed with an empty adsorption trap and an open loop, to make sure that no trace organics remained in the system.

Table 1: List of VOCs present in saliva (S) and/or Tongue coating (T) samples with their numbers used in the Figures and their corresponding retention times (Time).

No.	Compound	S/T	Time
1. S-compounds			
1	2-methyl-tio-propane	S	407
4	methanethiol	ST	431
5	mercapto-acetic acid	ST	435
8	methylsulphide	ST	465
9	propane-thiol	ST	493
23	methylthiopropane	T	654
25	thiocyanic acid	ST	666
26	ethanethioic acid-S-ME	ST	680
28	methylbenzotiophene	T	698
30	methylthiopropene	ST	733
34	dimethyldisulphide	ST	772
59	dimethyltrisulphide	ST	1262
66	benzenecarbothiotic acid	T	1458
78	methanesulfonylazide	T	1784
2. Acids			
16	acetic acid	S	577
27	2-methylpropanoic acid	ST	691
40	butyric acid	T	864
3. Alcohols			
7	1-propoxy-2-propanol	ST	459
15	2,4 pentanediol	ST	558
4. Phenyl compounds			
13	pyran compound	ST	536
17	pyran compound	S	604
20	pyran compound	S	635
47	methyl-pyridine	S	1007
48	ethylbenzene	ST	1038
49	pyran compound	S	1042
50	dimethylbenzene	ST	1058
54	dimethylbenzene	ST	1110
58	ethenyl-pyridine	S	1245
60	phenol	ST	1274
64	limonene	ST	1430
67	p-cresol	ST	1476
71	dimethylphenol	T	1557
74	benzaldehyde compound	ST	1711
75	naphtalene	ST	1732
79	indole	ST	1904
80	skatole	T	2085
83	phenol compound	ST	2262

5. Alkanes/alkenes			
10	2-methyl-propane	ST	500
11	dimethoxy-methane	ST	515
18	trichloro-ethane	ST	610
22	1 (1 ethoxyethoxy) propane	S	650
29	2-methyl-1-nitropropane	T	707
33	2,4 pentadienenitrile	S	764
6. Ketones/aldehydes			
39	2-hexanon	T	859
42	2-heptenal	T	902
52	2-heptanon	T	1082
57	6-methyl-2-heptanon	ST	1233
63	2-octanal	S	1402
7. Miscellaneous/unknown			
2	unknown	ST	413
3	diamine-compound	S	427
6	unknown	ST	447
12	hydroxylamino compound	S	520
19	unknown	T	615
21	1H-pyrrole compound	S	644
24	pyrrolidine	ST	660
31	unknown	S	748
32	furanone compound	ST	760
35	1H-pyrrole	ST	784
36	hydroxylamino compound	ST	789
38	unknown	S	846
41	hydroxylamino compound	ST	877
43	hydroxylamino compound	T	926
44	furan compound	ST	965
45	unknown	S	973
46	unknown	S	1002
51	imidazole compound	ST	1070
55	unknown	ST	1126
56	hydroxulamino compound	ST	1151
61	unknown	ST	1292
62	unknown	T	1307
65	unknown	ST	1445
68	unknown	S	1480
69	unknown	S	1487
70	unknown	ST	1514
72	furan compound	T	1582
73	furanon compound	S	1649
76	unknown	ST	1746
77	unknown	ST	1758
81	unknown	ST	2160
82	unknown	ST	2216
84	unknown	T	2295
85	unknown	S	2343
8. Internal standards			
14	bromochloromethane (IS 1)		555
37	1-chloro-2-bromopropane (IS 2)		828
53	1,4-dichlorobutane (IS 3)		1099

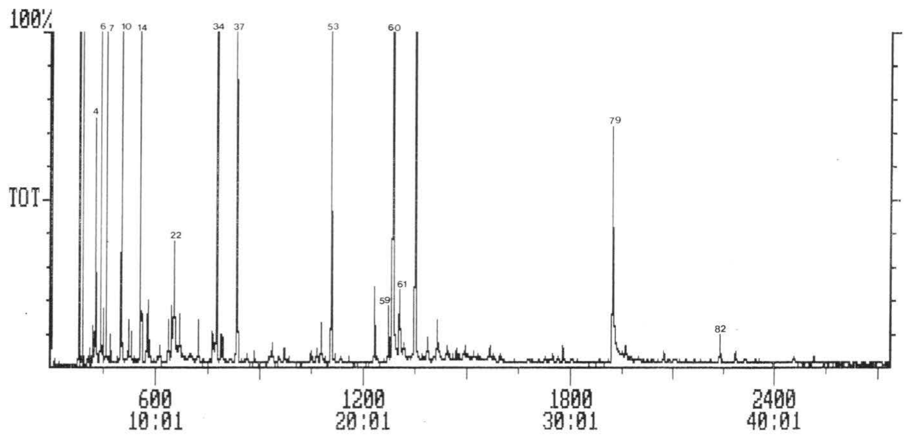


Fig. 2.:Total ion chromatogram of the VOCs of a saliva sample. x-Axis: scan number and retention time (min). Peak numbers indicate compound No., listed in Table 1.

Results

Quality control of the system is described by Ghoos and coworkers (13).

For the determination of the volatile compounds from saliva, 5 healthy volunteers without any complaints or oral malodour were studied. For the determination of the volatile compounds from tongue coating, samples were taken from 6 patients from the Leuven halitosis consultation (15).

Fig. 2 shows a chromatogram of a saliva sample. Compounds were identified on the basis of purity and fitted matches between the mass spectra generated by the ion trap detector and the spectra in the computer-based NBS-library (Environmental Protection Agency, National Institute of Health,Washington DC, USA). Some compounds remained unknown due to difficulties in mass spectrum interpretation.

Fig. 3 shows a chromatogram of a tongue coating sample.

After subtraction of the volatile background compounds, originating from Tenax GR and buffer solution, 85 compounds (IS included) remained.

These compounds are listed in Table 1. An attempt is made to classify these compounds in 8 categories according to their chemical nature. Additional characterization is done by appearance of each compound in saliva (S) and/or tongue coating (T) and by their retention time.

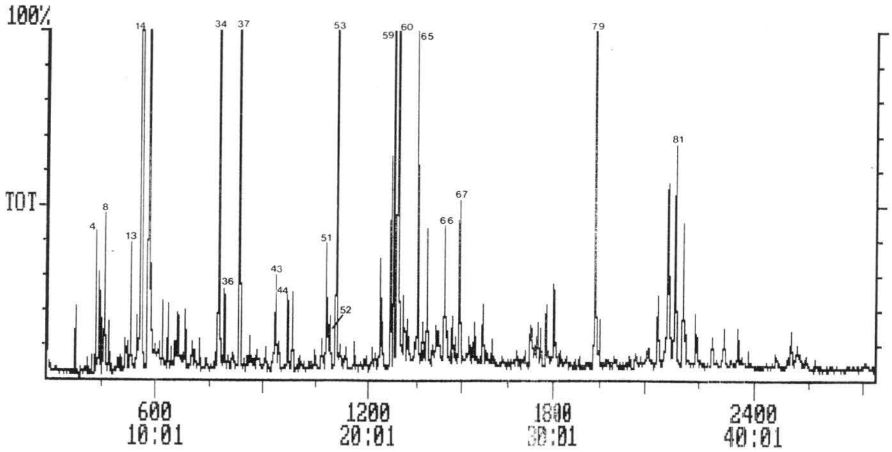


Fig. 3: Total ion chromatogram of the VOCs of a tongue coating sample. Labelling of axes and peaks as in Fig. 2.

Fig 4 A shows a chromatogram of a tongue coating sample. Fig. 4 B shows a chromatogram of the same tongue coating sample incubated with 5 mg of casein. Upon adding casein to the incubation system, different volatile compounds increased and new compounds appeared. Among them were 9 S-containing compounds. They are listed in Table 2 together with their retention times.

Table 2: List of S-containing VOCs which appear after the in vitro casein incubation of tongue coating, with their corresponding retention times (Time).

Compound	Time
2 ethylthio-ethanol	522
methyl-thio-butane	875
2,2 thiobis-ethanol	970
2 methyl-3-pentanethiol	1012
methyl propyl disulphide	1203
3 pentanethiol	1221
2 methyl-2-heptanethiol	1480
methyl (methylthio) methyl disulphide	1594
[(methylthio) methyl] benzene	2037

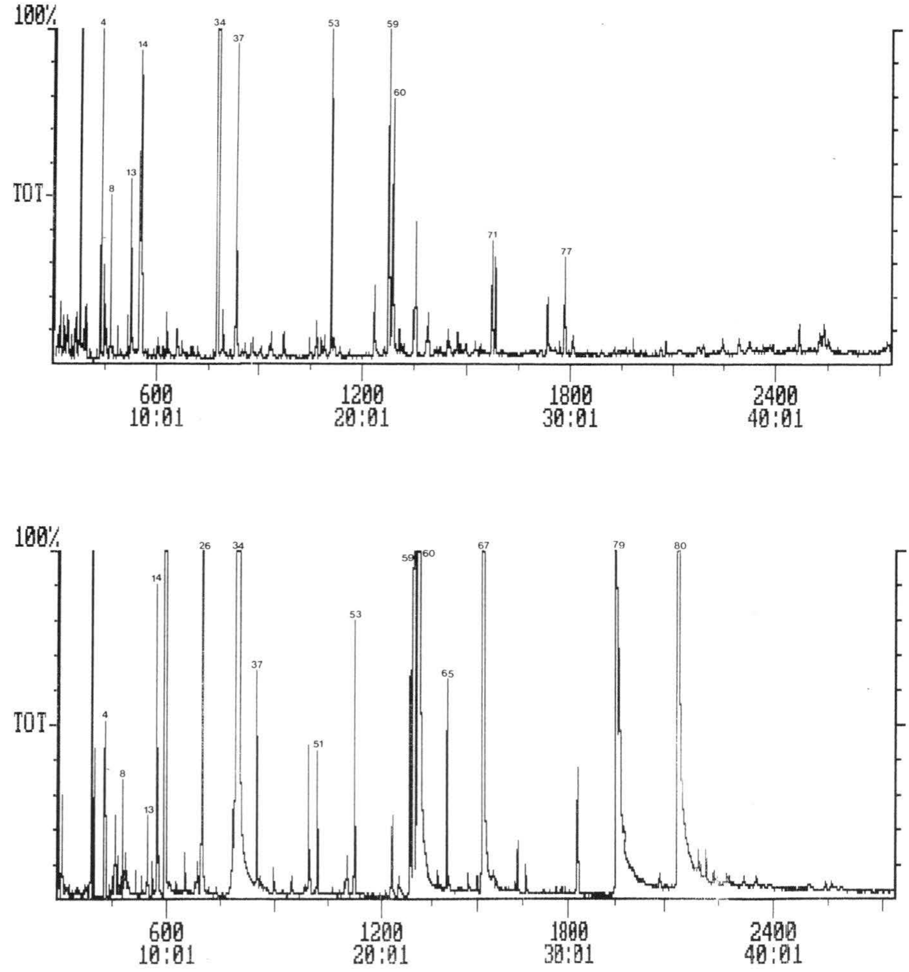


Fig. 4: Total ion chromatogram of the VOCs: (A) of tongue coating sample, (B) of tongue coating sample incubated in vitro with casein. Labelling of axes and peaks as in Fig. 2.

Discussion

In this study we validated a closed-loop trapping system for saliva and tongue coating samples. It is the first time that this system has been applied for the study of the formation of volatile compounds in the mouth. The rationale of this study is that in the last decade there is a growing interest in the mechanisms of oral malodour. Recent studies implicate the dorso-posterior surface of the tongue as the principal source of oral malodour (16). Some

S-containing bacterial fermentation products may even cause cell epithelium damage and systemic diseases (17).

A lot of work has been done on S-containing compounds since they were pointed out as the main contributors to oral malodour (1,18). The present study on analysis of volatile organic compounds is to be considered as an additional tool to gain insight into the total composition of oral malodour and even in the fermentation processes of the oral microbiota on different substrates. The authors are well aware of the fact that these analyses are just a first step. Therefore, maximal precaution has been given to the described method to minimize errors in analytical data. As the described system, i.e. closed-loop trapping thermodesorption-gas chromatography-ion trap detection, is very sensitive to external contamination, each step has been controlled carefully. Additionally, despite considerable attention in interpreting mass spectra, it was not possible to identify all compounds. With techniques such as chemical ionization and MS-MS, fewer compounds should remain unidentified. In this study, quantitative analysis of compounds was not carried out, due to the quasi impossible task of studying response factors of each compound versus internal standards. These internal standards however are of great importance in controlling the stability of the system and in supporting the reliability of the qualitative data. Attention should also be paid to the fact that equimolar quantities of different compounds can give different responses in the detector. Therefore this technique can only be used as a screening method for the determination of the volatile compounds. Nevertheless, some conclusions may already be drawn.

Besides the 13 S-containing compounds in normal tongue coating samples, 9 new S-containing compounds appeared after incubation with casein. These compounds are intermediary or end products of the breakdown of cysteine and methionine, two amino acids present in casein.

The increase of phenol and p-cresol is due to the breakdown of tyrosine, also present in casein (19).

The increase of indole and skatole is due to the breakdown of tryptophan (19).

This study aims to contribute substantially to better knowledge of metabolite formation in the oral cavity by determination of the volatile patterns of saliva and tongue coating samples.

In conclusion, the combination of an off-line preconcentration by means of a closed-loop trapping system followed by GC-ITD is a reliable method for screening volatiles in these samples on a qualitative basis.

With this technique it should be possible to gain more insight into the problem of oral malodour.

Acknowledgements

The authors acknowledge the excellent technical assistance of N. Gorris, A. Luypaerts, S. Rutten and L. Swinnen. B. Lepoudre is acknowledged for the preparation of the manuscript.

Part of the text and fig. 1 are a copy of the article, published in the Journal of Chromatography 665;333-345 (1994): Screening method for the determination of volatiles in biomedical samples by means of an off-line closed-loop trapping system and high-resolution gas chromatography-ion trap detection by Y. Ghoos, D. Claus, B. Geypens, M. Hiele, B. Maes and P. Rutgeerts.

Permission for publication was given by Elsevier Science, Amsterdam.

References

1. Tonzetich J. and Richter V (1964) Evaluation of volatile odoriferous components of saliva. *Archs oral Biol* 9:39-45.
2. Goldberg S., Kozlovsky A., Gordon D., Gelernter I., Sintov A. and Rosenberg M. (1994) Cadaverine as a putative component of oral malodour. *J Dent Res* 73:1168-1172.
3. Zlatkis A., Brasell R. and Poole C. (1981) The role of organic volatile profiles in clinical diagnosis. *Clin Chem* 27:789-797.
4. Manolis A. (1983) The diagnostic potential of breath analysis. *Clin Chem* 29:5-15.
5. Phillips M. and Greenberg J. (1992) Ion-trap detection of volatile organic compounds in alveolar breath. *Clin Chem* 38:60-65.
6. Preti G., Clark L., Cowart B., Feldman R., Lowry L., Weber E. and Young I. (1992) Non-oral etiologies of oral malodor and altered chemosensation. *J Periodontol* 63:790-796.
7. Marthinsen D. and Fleming S. (1982) Excretion of breath and flatus gases by humans consuming high-fiber diets. *J Nutr* 112:1133-1143.
8. Tomlin J., Lowis C. and Read N. (1991) Investigation of normal flatus production in healthy volunteers. *Gut* 32:665-669.
9. Hiele M., Ghoos Y., Rutgeerts P. and Vantrappen G. (1991) Influence of nutritional substrates on the formation of volatiles by the fecal flora. *Gastroenterology* 100:1597-1602.
10. Macfarlane G. and Cummings J. (1991) In: Phillips S., Pemberton J. and Shorter R. (Eds) *The large intestine: physiology, pathophysiology and disease.* Raven Press, New York; pp51-94.

11. Ghoos Y., Claus D., Geypens B., Hiele M., Maes B. and Rutgeerts P. (1994) Screening method for the determination of volatiles in biomedical samples by means of an off-line closed loop trapping system and high-resolution gas chromatography-ion trap detection. *J Chromatogr* 665:335-345.
12. Badings H., De Jong C. and Dooper R. (1985) In: Sandra P. and Bertsch W. (Eds) *Proceedings of the 6th international symposium on capillary chromatography* Huethig Verlag, Heidelberg, pp 666-678.
13. Ghoos Y., Hiele M., Rutgeerts P. and Vantrappen G. (1989) How to avoid water contamination in the thermodesorption cryofocussing technique. Application to ethane analysis in human breath by means of an ion trap detector (ITD). *J High Res Chromatogr* 12:739-741.
14. Belay N. and Daniels L. (1987) Production of ethane, ethylene and acetylene from halogenated hydrocarbons by methanogenic bacteria. *Appl Environ Microbiol* 53:1604-1610.
15. Delanghe G., Ghyselen J., Feenstra L. and van Steenberghe D. (1996) Experiences of a Belgian Multidisciplinary Breath Odour Clinic.. In: van Steenberghe D. and Rosenberg M. (Eds) *Bad breath: a multidisciplinary approach.* Leuven University Press, Leuven, Belgium, pp 199-208.
16. Miyazaki H., Sakao S., Katoh Y. and Takehara T. (1995) Correlation between volatile sulphur compounds and certain oral health measurements in the general population. *J Periodontol* 66:679-684.
17. Roediger W., Duncan A., Kapanisis O. and Millard S. (1993) Reducing sulphur compounds of the colon impair colonocyte nutrition: implication for ulcerative colitis. *Gastroenterology* 104:802-809.
18. Tonzetich J. (1971) Direct gas chromatographic analysis of sulphur compounds in mouth air in man. *Archs oral Biol* 16:587-597.
19. Barker H. (1981) Amino acid degradation by anaerobic bacteria. *Ann Rev Biochem* 50:23-40.

Characterization of Volatile Sulphur Compounds Production at Individual Crevicular Sites

J.M. Coil *

Introduction

Several investigators have shown that volatile sulphur compounds (VSC) emanate from the oral cavity. The reduction in intensity of putrefactive odour following thorough brushing of teeth and oral tissues demonstrates that the oral cavity is the principle source of breath malodour emitted via the mouth (1). It has also been shown by Tonzetich and co-workers (2, 3) that cellular elements in saliva are the principal source of substrate for the production of volatile sulphur compounds. Subsequently, Tonzetich introduced the gas chromatographic (GC) method employing a highly sensitive flame photometric detector (FPD) coupled with a GC unit for direct measurement of subnanogram quantities of VSC present in mouth air samples. Yaegaki and Sanada (4), employing GC analysis for sulphur compounds, have shown that VSC produced on the tongue surface in patients with periodontitis contributes 60% of the total sulphide content of mouth air. Reduction of bad breath from these patients was further accomplished by periodontal treatment which removed a significant portion of the VSC content from their air.

Rosenberg et al. (5), have reported on the Halimeter™ (Interscan Corp., Chatsworth, CA) a portable sulphide monitor capable of quantitative measurements of VSC in mouth breath. They found that there was a correlation between VSC in mouth air and the extent of periodontal breakdown, and that the sulphide monitor was highly correlated with organoleptic readings. De Boever and co-workers (6), measured VSC in mouth air using a Halimeter™ in patients with and without complaints of oral malodour. They found that patients with complaints had significantly higher sulphide concentrations in their breath. These investigators have evaluated VSC by three methods: organoleptic odour rating, GC analyses, and portable sulphide monitor measurements. To date, only GC analyses can distinguish and quantitate the various sulphide components.

---

* J.M. Coil, Department of Oral Medicine & Surgical Sciences, Faculty of Dentistry, University of British Columbia, 2199 Westbrook Mall, Vancouver, B.C., Canada V6T 1Z3.

The effect of therapy on volatile sulphur content of mouth air was first demonstrated by Tonzetich and Spouge (7). Their mouth air studies indicated that levels of VSC are considerably increased in the mouths of persons with periodontitis. In their study on twenty subjects suffering from periodontitis, the concentration of H_2S and CH_3SH correlated with the incidence and depth of periodontal pockets greater than 3 mm. Furthermore, the levels of both these compounds were markedly reduced following curettage and corrective periodontal surgery. Yaegaki and Sanada (4) reported that total sulphide content of mouth air was reduced in patients with periodontitis after tongue scraping had been performed.

Characterization of volatile sulphur compounds at crevicular sites was first reported by Rizzo (8). Using lead acetate impregnated filter paper strips, he reported a positive correlation between the amount of H_2S produced in the gingival crevice and the depth of corresponding periodontal pockets. Solis-Gaffar et al (9) measured the levels of the H_2S production in gingival crevicular fluid (GCF) and concluded that the levels of H_2S correlated with both the gingival index and the crevicular fluid volume. These investigators measured H_2S after each filter paper strip containing GCF was incubated with L-cysteine for 3 days.

One additional study, by Tonzetich and McBride (12), examined VSC production by pathogenic and non-pathogenic strains of oral Bacteroides. They found that pathogenic strains produced copious amounts of VSC compared to non-pathogenic strains. It is noteworthy that such pathogenic strains were typically found in deeper periodontal sites.

Our laboratory reported on the non-invasive direct measurement of VSC production at gingival sulci, in relation to periodontal involvement (10, 11). The main studies that I was involved in were i) to collect, analyze and compare the VSC components in gingival sulci and mouth air, and to determine the level of VSC production in the gingival crevice in relation to periodontal involvement, and ii) to study the collagen breakdown products in gingival crevicular fluid in healthy and affected periodontal sites.

Materials and Methods

Because it is impossible to obtain sufficiently large crevicular air samples for direct analyses, it was necessary to use Tenax-GC traps to pre-trap and concentrate the volatiles emanating from the gingival sites. Tenax traps were constructed from 7 mm OD x 75 mm lengths of aluminium tubing, coated on the inside with teflon, packed with 0.7 g of Tenax-GC, and stoppered with

glass wool end plugs. The same methodology for collection and concentration of volatile organic compounds from human mouth air has been previously described (13). Briefly, collection of volatiles was effected by use of Tenax trap which was placed between a vacuum pump and the subject's oral cavity.

Volatile collection from crevicular sites was effected by isolating and sampling from individual periodontal sites. A small section of micropolyethylene tubing (Intramedic™), 0.76 mm ID x 1.22 mm OD was held against the buccal surface of the tooth at the level of the gingival margin using dental floss interproximally. A soft acrylic denture liner, Coe-soft (Coe™), was fitted over the tooth and its adjacent tubing. After the material hardened, the impression was removed from the mouth and epoxy resin was used to cement the tubing as it exited the impression to the external portion of the acrylic. This created a closed system. Finally, the portion of tubing at the gingival margin was removed, creating future inlet and outlet ports for sampling. Each custom sampling device had a 60 cm outlet port of tubing extend from the crevicular site to a Tenax trap, while a 15 cm inlet port of tubing extended from the collection device to ambient air.

These special devices for collection of volatiles were constructed for each individual. They were prepared after the subjects provided mouth air samples for GC analysis. The collection device was placed back in the mouth and the exit port tubing was connected via tygon tubing to a Tenax trap cooled to -55°C in dry ice. The other end of the trap was connected to a peristaltic pump, and crevicular air was removed at a rate of 0.60 ml/min. Only one device was required per site which allowed for repetitive sampling.

Gas chromatographic analysis of crevicular samples was performed as follows. Immediately after collection; the compounds concentrated on Tenax traps were desorbed at 120°C using a modified Bendix Flasher coupled to the GC unit and analyzed by GC. The separations of VSC were performed on a Tracor 550 Gas Chromatograph using a 7.315 x 3.2 mm fluorinated ethylene phosphoric acid on 30-40 mesh Teflon. The temperature of the column and detector were 70°C and 150°C respectively.

Results

The identification of sulphide components was based upon their retention times. The chromatographic pattern exhibited sulphide peaks and retention times (Rt) as follows: hydrogen sulphide, H_2S (Rt: 115-130 sec.); methyl mercaptan, CH_3SH (Rt: 190-205 sec.); dimethyl sulphide, $(CH_3)_2S$ (Rt:

275-310 sec.); dimethyl disulphide, $(CH_3S)_2$ (Rt: 390-425 sec.). For sulphide peaks that were below the threshold quantitation level setting of the detector (µ100mV/sec), extrapolation calculations were performed using peak weights of known peak areas and corresponding ng quantities.

Qualitative and quantitative differences were found in compositions of mouth air and crevicular air samples. GC profiles for Tenax-trapped mouth air were reproducible and exhibited peaks corresponding to H_2S, CH_3SH, $(CH_3)_2S$ and $(CH_3S)_2$. A sample profile for both mouth and crevicular air shows that while both chromatograms exhibit profiles for H_2S, CH_3SH and $(CH_3)_2S$, the crevicular air sample shows significantly more CH_3SH and $(CH_3)_2S$ than the mouth air sample, demonstrating the composition of VSC from different sources. Another example compares profiles of Tenax-trapped crevicular air collected from two subjects. Whereas both chromatograms exhibit profiles of H_2S, $(CH_3)_2S$ and $(CH_3S)_2$, one chromatogram shows a prominent peak for CH_3SH, whose concentration exceeds that of H_2S.

Pilot studies in our laboratory examined the presence of sulphur containing compounds in mouth and crevicular air of periodontally involved individuals. While H_2S was consistently recovered from both sources, CH_3SH and $(CH_3S)_2$ presence differed in crevicular and mouth air samples. Evaluation of the ratios of VSC detemined in mouth air and crevicular air revealed that these ratios differed in mouth and crevicular air.

A study was done to compare VSC content of mouth air to crevicular air; and to examine VSC in shallow (< 3 mm) versus deep (> 4 mm), and noninflamed versus inflamed (bleeding on probing) periodontal sites. Seventeen patients (12 male and 5 female, aged 24-67 yrs), were selected on the basis of having no previous periodontal treatment or antibiotic medication within the past six months and having no known systemic disease. Pocket depths were measured with a marked probe to the nearest millimeter. Periodontal sites were separated into control and test groups based upon pocket depth measurements and bleeding on probing status.

Results indicated that, as previously, there were differences in the frequency and in concentrations of VSC in mouth air and crevicular air from control and test sites. In both mouth and crevicular air, H_2S was the most frequently observed VSC in all four categories. The occurrence of both CH_3SH and $(CH_3)_2S$ was similar or slightly greater in crevicular air vs. mouth air. There appeared to be no great differences in frequency between control and test groups for both of these sulphides in mouth air and crevicular air. However $(CH_3S)_2$ did not appear to predominate in either the control or test groups.

Analyses were performed on sulphide ratios in crevicular air comparing deep versus shallow, and noninflamed versus inflamed periodontal sites. A similar

trend was found for both the ratios of methyl mercaptan to H_2S and all methyl sulphides to H_2S. Deeper crevicular sites exhibited higher sulphide to H_2S ratios than the shallow sites. In particular, the CH_3SH to H_2S ratio of the test group was somewhat greater than the control ($p < 0.10$). Similarly, the ratio of CH_3SH to H_2S was greater in inflamed sites versus noninflamed sites ($p < 0.05$).

Summary

In summary, our studies have shown that 1) sufficient amounts of VSC can be collected from the gingival crevice for GC analysis, 2) that both qualitative (the presence or absence of sulphides) and quantitative (the sulphide ratios) differences exist between mouth air and crevicular air, 3) hydrogen sulphide was the most dominant sulphide observed in crevicular air, 4) methyl mercaptan, was the most frequently observed methyl sulphide in crevicular air, 5) the CH_3SH to H_2S ratio in crevicular air was significantly higher at inflamed and deep than noninflamed and shallow periodontal sites. Also to summarize the second study, using monoclonal and polyclonal antibodies to collagen and HPLC measurement for hydroxyproline, an increase in collagen breakdown products were found in inflamed versus noninflamed periodontal sites. This finding parallels the investigation by Johnson, Yaegaki and Tonzetich (14), who found that VSC increases collagen solubility which make it more susceptible to enzymatic degradation and contribute to increased destruction of collagen.

References

1. Sulser G.F., Brening R.H. & Fosdick L.S. (1939) Some conditions that affect the odor concentrations of the breath. *J Dent Res* 18:355-359.
2. Tonzetich J. & Richter V.J. (1964) Evaluation of volatile odoriferous compounds of saliva. *Archs oral Biol* 9:39-45.
3. Tonzetich J. & Kestenbaum R.C. (1969) Odour production by human salivary fractions and plaque. *Archs oral Biol* 14:815-827.
4. Yaegaki K. & Sanada K. (1992) Volatile sulfur compounds in mouth air from clinically healthy subjects and patients with periodontal disease. *J Periodont Res* 27:233-238.
5. Rosenberg M., Bosy A., Kulkarni G.V. & McCulloch C.A.G. (1991) Reproducibility and sensitivity of oral malodor measurements with a

portable sulphide monitor. *J Dent Res* 70:1436-1440.
6. De Boever E.H., De Uzeda M. & Loesche W.J. (1994) Relationship between volatile sulfur compounds, BANA-hydrolyzing bacteria and gingival health in patients with and without complaints of oral malodor. *J Clin Dent* 4:114-119.
7. Tonzetich J. & Spouge J.D. (1979) Effect of periodontal therapy on volatile sulphur content of mouth air. *J Dent Res* 58:175 (abstract).
8. Rizzo A.A. (1967) The possible role of hydrogen sulfide in human periodontal disease. I. Hydrogen sulfide production in periodontal pockets. *Periodontics* 5:233-236.
9. Solis-Gaffar M.C., Rustogi K.N. & Gaffar A. (1980) Hydrogen sulfide production from gingival crevicular fluid. *J Periodontol* 51:603-606.
10. Coil J. & Tonzetich J. (1985) Volatile sulphur production at healthy and disease gingival crevice sites. *J Dent Res* 64:306 (abstract).
11. Coil J.M. & Tonzetich J. (1992) Characterization of volatile sulphur compounds production at individual gingival crevicular sites in human. *J Clin Dent* 3:97-103.
12. Tonzetich J. & McBride B.C. (1981) Characterization of volatile sulphur production by pathogenic and non-pathogenic strains of oral Bacteroides. *Archs oral Biol* 26:963-969.
13. Tonzetich J., Coil J. & Ng W. (1991) Gas chromatographic method for trapping and detection of volatile organic compounds from human mouth air. *J Clin Dent* 2:79-82.
14. Johnson P.W., Yaegaki K. & Tonzetich J. (1992) Effect of volatile thiol compounds on protein metabolism by human gingival fibroblasts. *J Periodont Res* 27:553-561.

The Effect of Methyl Mercaptan on the Enzymatic and Immunological Activity Leading to Periodontal Tissue Destruction

L. G. Ratkay, J. Tonzetich and J.D. Waterfield *

Introduction

Bacterial pathogens have been shown to be the etiological agents involved in the development of periodontitis. *Porphyromonas gingivalis* is considered to be a major etiological agent in adult periodontitis (1). *Actinobacillus actinomycetemcomitans* has been associated with localized juvenile periodontitis, as well as acute adult periodontitis (2), whereas *Prevotella intermedia*, fusiforms and *Treponema* species are implicated in different forms of gingivitis (3). These organisms, as well as their secreted products, can exert a direct toxic effect on host cells and/or an indirect effect by activating host immune responses which in turn may lead to pathological manifestations. One such secreted compound, methyl mercaptan, a volatile sulphur compound (VSC), is a by-product of protein metabolism by periodontopathic bacteria and a major component of oral malodour.

Several studies have demonstrated the pathological importance of volatile sulphur compounds on the integrity of oral mucosa and cellular metabolism (4, 5, 6).

As the level of these volatile sulphur compounds are elevated in periodontitis (7) it has been suggested that they may be a contributing factor in the enzymatic and immune reactions leading to tissue degradation.

Effect of methyl mercaptan on interleukin-1 production

The pathogenesis of periodontitis is characterized by a marked reduction of collagen in the affected gingival connective tissues (8). It is generally believed that increased intra- and extra-cellular collagenolysis "in situ" results in part from an activation of the immune system resulting in the production of cytokines which promote production and conversion of the relevant

* L.G. Ratkay, J. Tonzetich and J.D. Waterfield, Department of Oral Biology, Faculty of Dentistry, University of British Columbia, 2199 Westbrook Mall, Vancouver, B.C., Canada V6T 1Z3.

proteolytic proenzymes to an active form. Charon et al (9) have reported a significant increase of one such cytokine, interleukin-1(IL-1), in gingival fluids from inflamed sites in patients suffering from periodontitis and demonstrated that production of this cytokine correlated with the severity of disease. Interleukin- 1 is a member of a family of closely related proteins whose pathophysiological role in tissue destruction is currently under investigation. Although few *in situ* studies have been carried out, *in vitro* findings suggest that IL-1 is a pivotal cytokine in the inflammatory response. It has been demonstrated that IL-1:

a) is directly involved in the induction of monocyte chemotactic protein production. These proinflammatory inducible proteins recruit and activate the major cells involved in inflammation (10)
b) mediates expression of E-selectin, cellular adhesion molecules found on the surface of endothelial cells that initiate rolling contacts with PMNs and together with integrin/ICAM-1 allow migration of the PMNs through the walls of blood vessels (11).
c) initiates types II, III and IV hypersensitivity reactions in the tissues.
d) is directly involved in the activation of osteoclasts responsible for bone resorption, as well as promoting bone resorption by a PGE2 mediated pathway using adenosine 3', 5' cyclic monophosphate (cAMP) as a second messenger (12).
e) is involved in the production of neutral proteinases (13).

In the present study, mononuclear cell cultures from tonsillar tissue were stimulated with LPS in the presence or absence of 13.7 ng methyl mercaptan/ml of CO_2/air head space as previously reported (6). After 48 hr of incubation serial dilutions of the supernatants from these cultures were added to 2 X 10^4 D10.G4.1 cells (an IL-1-dependent cell line) together with 2.5 µg/ml concanavalin A. After a further 48 hr of incubation the latter cultures were pulsed with 1.0 µCi [^{3}H]-thymidine. The IL-1-dependent cellular proliferation was determined by liquid scintillation counting of the radionucleotide uptake, and the IL-1 content was derived from a dose response curve for recombinant IL-1. It can be readily perceived from Fig. 1 that the supernatants derived from cells exposed to methyl mercaptan induced a two-fold higher stimulation index than supernatants of control cell systems. This effect represents a 20-fold increase in the amount of IL-1 produced in the presence of methyl mercaptan. Methyl mercaptan alone was found to increase the production of IL-1 (data not shown).

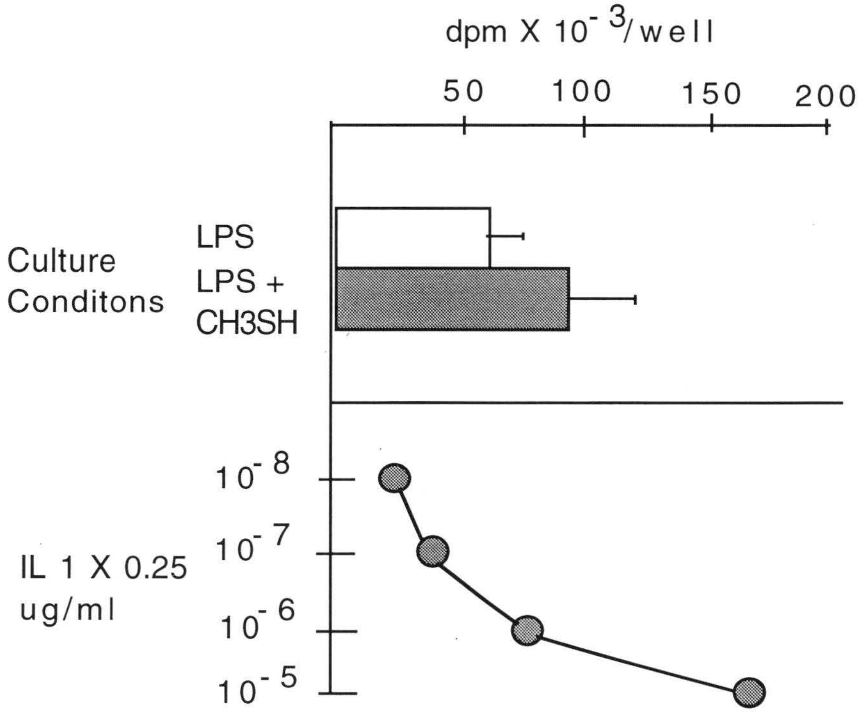


Fig. 1: The effect of methyl mercaptan on LPS-induction of IL-1 synthesis. The results represent the mean of the dpm of radionucleotide incorporation of triplicate cultures of D10.G4.1.

Effect of methyl mercaptan on PGE_2 and cAMP production by human gingival fibroblasts

There is strong evidence that the expression of matrix metalloproteinases by mononuclear cells is mediated through the PGE_2-cAMP-dependent pathway; a cascade of second messenger signaling events initiated by a primary stimulus such as LPS or cytokines. Hence, it was of interest to determine the effect of methyl mercaptan on PGE_2 and cAMP production by cells other than leucocytes in this case the human gingival fibroblasts (HGF)-components of the connective tissue. In this series of experiments the HGF cells were cultured according to the method of Brunette et al. (14) in which cells (10^5 from 6-9 passaged cell lines) were plated on 24 well Falcon plates. After 24 hour incubation, the complete Dulbecco's Modified Eagles Medium (DMEM) was removed from the attached cells and replaced with the same media containing either LPS or IL-1, or both, and exposed to methyl mercaptan as

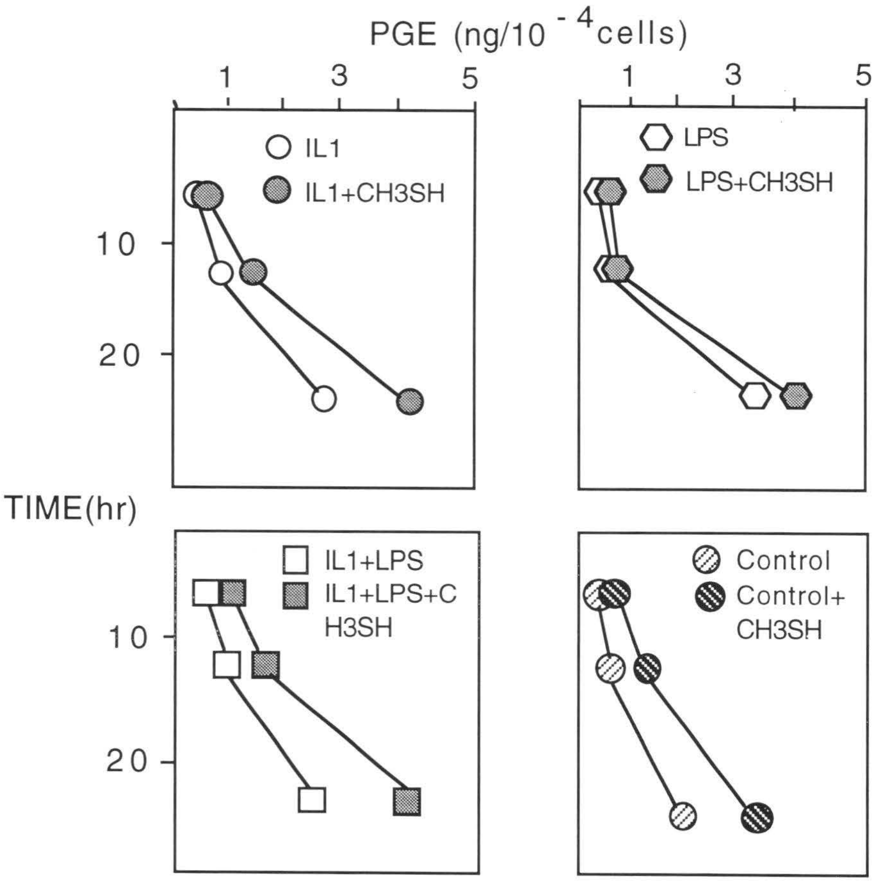


Fig. 2: The effect of methyl mercaptan on PGE2 production by human gingival fibroblasts in the presence or absence of recombinant IL-1 and LPS. The values represent the means of ELISA optical density measurements.

described above. After 6, 12 and 24 hr the supernatants were harvested and varying dilutions tested in an ELISA assay as described by Ratkay et al. (15) with a PGE_2 specific antibody. The concentrations of PGE_2 were obtained from standard curves derived from serial dilutions of the corresponding antigen. Fig. 2 demonstrates that methyl mercaptan stimulated PGE2 production alone, or in the presence of IL-1, or in the presence of both IL-1 and LPS. However, PGE_2 levels were not significantly altered in the presence of both LPS and methyl mercaptan. A similar experimental design was used in the experiments ascertaining the effect of methyl mercaptan on cAMP production in which the actual measurement of cAMP in the cell supernatants

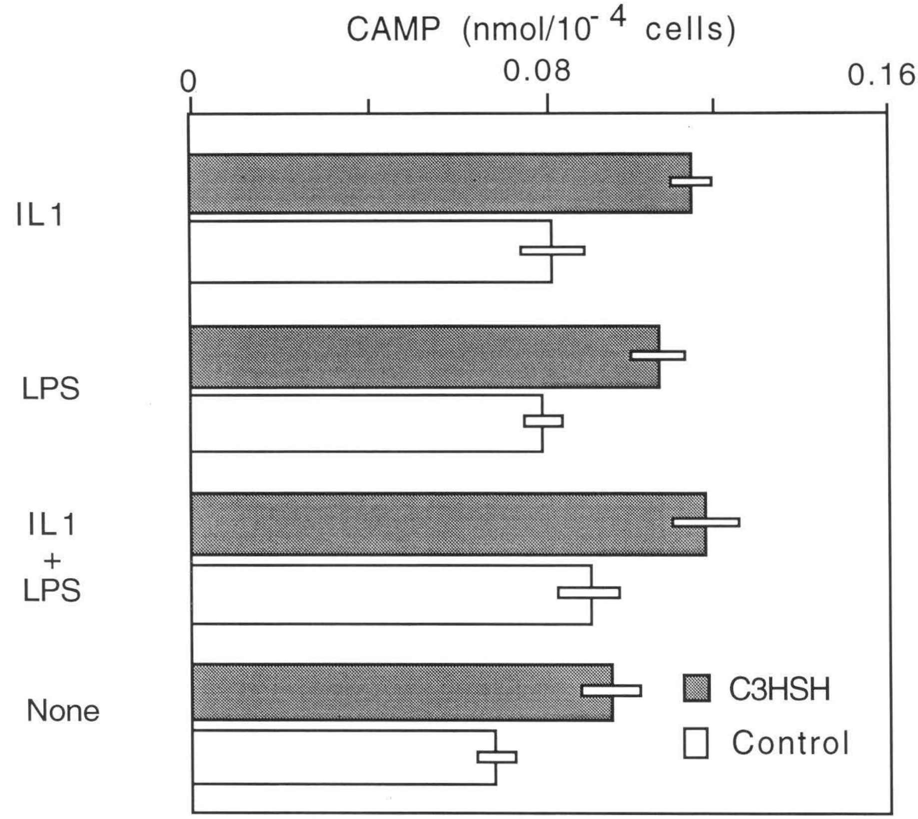


Fig. 3: cAMP production by methyl mercaptan treated human gingival fibroblasts in the presence or absence of IL-1 and LPS. The results are the means ± SD of ELISA optical density measurements.

was carried out using a radioimmunoassay (RIA). In the latter instance the supernatants were heated at 90-100°C for 3-4 min in a boiling water-bath, immediately cooled at 4°C then extracted in ethanol before being quantified by RIA using a standard curve. As it can be seen in Fig. 3, all three agents alone, or in combination, significantly elevated cAMP production by human gingival fibroblasts over a 24 hr period. Methyl mercaptan alone increased the amount by 37%, while IL-1, LPS- and IL-1/LPS-supplemented cultures yielded 33, 23, and 40% higher cAMP content than the controls. Simultaneous exposure cultures to methyl mercaptan and IL-1, LPS, IL-1/LPS further increased the cAMP contents by 77, 70 and 78% respectively. A similar radioimmunoassay was carried out on cultures of human gingival fibroblasts incubated for up to 24 hr in the presence or

absence of methyl mercaptan, and on cultures supplemented with supernatants from methyl mercaptan-exposed mononuclear cell cultures that exhibited increased IL-1 production above that induced by LPS alone. The results of this study (data not shown) were in close agreement with our above findings.

Effect of methyl mercapatan on collagenase production

The interstitial collagenase is believed to play an important role in the initiation of collagen degradation (16). The enzyme is secreted as procollagenase, which can be activated by serine proteases (plasmin, kallikrein, lysosomal

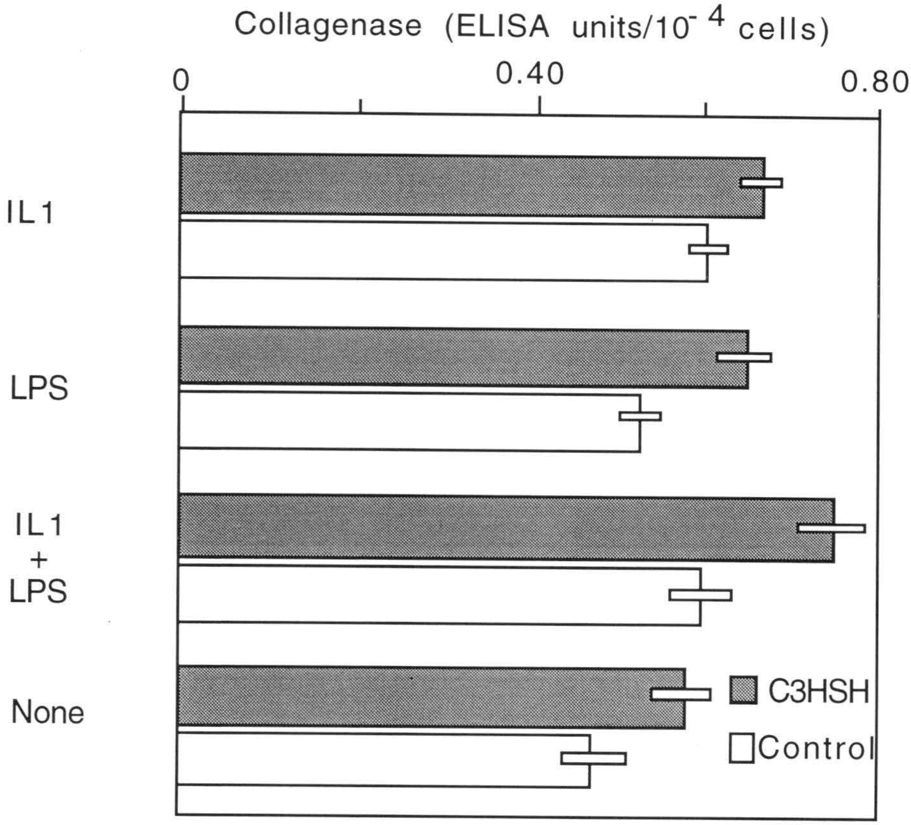


Fig. 4: The effect of methyl mercaptan on collagenase production by human gingival fibroblasts in the presence or absence of IL-1 and LPS. The data represent the means ± SD of ELISA optical density units.

cathepsin B), plaque, and periodontitis associated bacteria and is elevated in inflamed periodontal tissue (17). In our study the effect of methyl mercaptan alone, or in combination with IL-1, LPS and IL-1/LPS was examined under the same culture conditions as described above. The production of collagenase was measured by ELISA and presented in optical density units, as purified preparations of collagenase were not able to establish standard curves. It can be seen from Fig. 4 that in comparison to the non-stimulated controls, methyl mercaptan alone increased the production of collagenase by 44%. This value approximates that of IL-1, LPS and IL-1/LPS which under similar experimental conditions increased the levels of collagenase by 49, 25, and 39% respectively. In combination with these stimulators, methyl mercaptan enhanced collagenase production by 62, 57 and 74%. Thus, it is apparent that methyl mercaptan alone, or in combination with IL-1 or LPS induced a strong stimulatory effect on the production of collagenase by human gingival fibroblasts.

Effect of methyl mercaptan on cathepsin B, cathepsin G and elastase production

Cathepsins are involved in both the intracellular and extracellular pathways of collagen degradation. Cathepsin B, an intracellular lysosomal thiol-dependent proteinase, was evaluated in HGF cultures in the presence and absence of methyl mercaptan as described above. The activity of cathepsin B was determined in a fluorometric assay using Z-Arg-Arg-MLA as a substrate. Cathepsin G, an extracellular aspartate proteinase and elastase, an extracellular serine proteinase, derived from polymorphonuclear leucocytes, were introduced in the presence or absence of methyl mercaptan. In the latter case the enzymatic activities were measured using the chromogenic peptide substrates N-Suc-Ala-Ala-Pro-Phe-pNa (Cathepsin G) and Meo-Suc-Ala-Ala-Pro-Val-pNa (Elastase).

Table I demonstrates that the production of cathepsin B was increased by 25% in fibroblasts cultures that were continuously exposed to methyl mercaptan. An increase of the same magnitude was observed in cultures supplemented with 6 mM cysteine confirming the thiol-dependency of cathepsin B. Table I also demonstrates that methyl mercaptan suppressed cathepsin G activity while having no effect on elastase.

Table 1: The effect of methyl mercaptan on the activity of cathepsin B, cathepsin G and elastase

Test	% Change in activity [(+)]		
System	Cathepsin B	Cathepsin G	Elastase
CH3SH (10 ng/ml)	+ 24.6	-26.0	+ 1.0
Cysteine (6 mM)	+ 29.4	-29.0	- (*)

(+) The % change in activity values were calculated in relation to comparable control cultures to which no agents were added
(*) not determined

Discussion

Over the past two decades there has been a marked increase in our knowledge of the etiopathogenesis of periodontitis. Significant evidence has accumulated suggesting that the matrix neutral metalloproteinases are involved in tissue destruction in which collagenases appear to play a major role. Interstitial collagenases are secreted by fibroblasts, epithelial cells, polymorphonuclear leucocytes and other cells and cleave the extracellular collagen into ¾-¼ fragments. These enzymes are secreted as procollagenases and are probably activated locally by serine proteases, plaque or periodontopathic bacteria (17). In contrast, latent interstitial collagenases are not activated by such proteases and thus appear to resemble neutrophil procollagenase (18). Collagenase activity and latent collagenase activity are elevated in inflamed periodontal tissue, crevicular fluid and whole saliva, and the activity decreases after periodontal treatment. Recent findings have suggested that cytokines such as IL-1 are capable of inducing the expression of these matrix metalloproteinases. As IL-1 is a pivotal cytokine for activation of the immune system, and is found in pathophysiological concentrations in gingival crevicular fluid, its role in tissue destruction cannot be underestimated. Such pyrogenic cytokines as IL-1 appear to elicit their effect in mononuclear cells through the activation of the PGE_2-cAMP dependent pathway.

Our findings indicate that methyl mercaptan, a degradation product of S-amino acid-containing proteinaceous substrates, is effective in augmenting the LPS activation of IL-1 production by mononuclear cells. We propose a cascade of events that eventually results in the decreased collagen found in the human gingival fibroblast cultures. In the presented model the enhanced IL-1 production by the mononuclear cells trigger HGF to produce increased

levels of PGE_2 and cAMP. This in turn, results in a stimulation and increased production of active neutral proteinases such as plasminogen activator, collagenase, and elastase which contribute to the increased rate of collagen degradation. This study also demonstrates that methyl mercaptan can act both directly or in synergism with IL-1 on the human gingival fibroblasts by enhancing the secretion of PGE_2, cAMP and collagenase. However, this study did not discern whether the elevation of cAMP and procollagenase is attributed directly to methyl mercaptan or whether PGE_2 is an intermediate step in the pathway. In addition, our study shows that methyl mercaptan significantly augmented the activity of cathepsin B, moderately suppressed cathepsin G and had no effect on elastase.

From this study it is not clear whether the activation of fibroblasts in inflamed periodontal tissue is involved in the pathogenesis of periodontitis. Collagenase activity derived from polymorphonuclear leucocytes is found in the crevicular fluid of inflamed tissue and appears to correlate with disease activity (19). However, the determination of leucocyte-derived collagenase in gingival crevicular fluid does not necessarily represent what is occurring in the connective tissue. It is conceivable that fibroblast collagenase production also correlates with disease acitivity. Our findings suggest that methyl mercaptan contributes to tissue destruction in periodontitis by exerting both an indirect effect through mononuclear cells as well as a direct effect through its interaction with connective tissue fibroblasts.

References

1. Slots J. and Genco R.J. (1984) Black-pigmented *Bacteroides* species, *Capnocytophaga* species, and *Actinobacillus actinomycetemcomitans* in human periodontal disease: Virulence factors in colonization, survival, and tissue destruction. *J Dent Res* 63:412-421.
2. Genco R.J., Zambon J.J. and Christersson L.A. (1988) The origin of periodontal disease. *Adv Dent Res* 2:245-254.
3. Listgarten M.A. (1965) Electron microscopic observations of the bacterial flora of acute necrotizing ulcerative gingivitis. *J Periodontol* 36:328-339.
4. Johnson P.W., Ng W. and Tonzetich J. (1992) Modulation of human gingival fibroblast cell metabolism by methyl mercaptan. *J Periodont Res* 27:476-483.
5. Tonzetich J. (1994) Preface. In: Rosenberg M. (Ed) *Bad Breath: Research Perspectives.* Ramot Publishing, Tel-Aviv, pp Xi-XViii.

6. Johnson P.W., Ng W. and Tonzetich J. (1992) Effects of volatile thiol compounds on protein metabolism by human gingival fibroblasts. *J Periodont Res* 27:553-561.
7. Tonzetich J. and Spouge J.D. (1979) Effect of periodontal therapy on volatile sulfur content of mouth air. *J Dent Res 58:*175 (abstract).
8. Schroeder H.E., Munzel-Pedrazzoli S. and Page R.C. (1973) Correlated morphometric and biochemical analysis of gingival tissues in early chronic gingivitis in man. *Archs oral Biol* 18:899-923.
9. Charon J., Luger T.A., Mergenhagen S.E. and Oppenheim J.J. (1982) Increased thymocyte-activating factors in human gingival fluid during gingival inflammation. *Infect Immun* 38:1190-1195.
10. Koch A.E., Kundel S.L., Harlow L.A., Johnson B., Evanoff H.L., Haines G.K., Burdik M.D., Pope R.M. and Strieter R.M. (1992) Enhanced production of monocyte chemoattractant protein-1 in rheumatoid arthritis. *J Clin Invest* 90:772-779.
11. Crawford J.M. and Wanatabe K. (1994) Cell adhesion molecules in inflammation and immunity: Relevance to periodontal diseases. *Crit Rev in Oral Biol and Med* 5(2):91-123.
12. Saito S., Ngan P., Saito M., Lanese R., Shanfeld J. and Davidovitch Z. (1990) Effects of cytokines on prostaglandin E and cAMP levels in human periodontal ligament fibroblasts in vitro. *J Dent Res* 69:1456-1462.
13. Giovine F.S. di and Duff G.W. (1990) Interleukin 1: the first interleukin. *Immunol Today* 11:13-16.
14. Brunette D.M., Melcher A.H. and Moe H.K. (1976) Culture and origin of epithelium-like and fibroblast-like cells from porcine periodontal ligament explants and cell suspensions. *Archs oral Biol* 21:393-400.
15. Ratkay L.G., Tonzetich J. and Waterfield J.D. (1991) Antibodies to extracellular matrix proteins in the sera of MRL-lpr mice. *Clin Immunol Immunopath* 59:236-245.
16. Kryshtalskyj E., Sodek, J. and Ferrier J.M. (1986) Correlation of collagenolytic enzymes and inhibitors in gingival crevicular fluid with clinical and microscopic changes in experimental periodontitis in the dog. *Archs oral Biol* 31:21-31.
17. Uitto V-J. and Raeste A-M. (1978) Activation of latent collagenase of human leukocytes and gingival fluid by bacterial plaque. *J Dent Res* 25:135-142.
18. Sorsa, T., Uitto V-J., Suomalainen K., Vauhkonen M. and Lindy S. (1988) Comparison of interstitial collagenase from human gingiva, sulcular fluid and polymorphonuclear leukocytes. *J Periodont Res* 23:386-393.

19. Ganghar S., Overall C.M., McCulloch C.A. and Sodek J. (1990) Identification of polymorphonuclear leukocyte collagenase and gelatinase activities in mouthrinse sample: correlation with periodontal disease activity in adult and juvenile periodontitis. *J Periodont Res* 25:257-267.

Effects of Methyl Mercaptan on Human Gingival Fibroblast Shape, Cytoskeleton and Protein Synthesis and the Inhibition of its Effect by Zn^{++}

D.M. Brunette, Y. Ouyang, J. Glass-Brudzinski and J. Tonzetich[*]

It is well established that volatile sulphur compounds (VSC) are the primary molecules responsible for oral malodour, mainly produced by the action of oral anaerobic Gram-negative bacteria, on sulphur containing proteinaceous substrates in saliva (1). A number of correlative studies have associated VSC with periodontal inflammatory diseases (2, 3, 4). For example VSC concentration in putrefying saliva correlated with the severity of periodontal involvement, but after corrective periodontal therapy VSC levels declined (3). Similarly, a comparison between pathogenic and non-pathogenic Bacteroides strains showed that pathogens produced 10 fold more VSC, especially CH_3SH and $(CH_3S)_2$, than the non-pathogens suggesting that VSC may be involved in the pathogenesis of tissue destruction (5).

One approach to controlling VSC levels and thus oral malodour has been the use of mouthrinses containing Zn^{++} ions. Zn^{++}, an essential tissue element, plays a significant role in many biological processes but can have toxic effects at high concentrations. Zn^{++} can reverse the effect of some VSC produced effects on oral tissues. For example, Zn^{++} reversed the effect of VSC-induced increase of porcine sublingual permeability to prostaglandin E and endotoxin (6). However, the ability of Zn^{++} to reverse other biological effects of VSC is largely unknown.

In this study the effects of methyl mercaptan (CH_3SH), the most potent VSC in terms of producing oral malodour, on cell proliferation, migration, shape, cytoskeletal distribution, proline transport, and protein synthesis are reported. In addition it was found that Zn^{++} could block the inhibitory effect of VSC on protein synthesis.

---

* D.M. Brunette, Y. Ouyang, J. Glass-Brudzinski and J. Tonzetich, Department of Oral Biology, Faculty of Dentistry, University of British Columbia, 2199 Westbrook Mall, Vancouver, B.C., Canada V6T 1Z3.

Materials and Methods

Cell Culture

Human gingival fibroblasts (HGF) between the fourth and twelfth subculture, isolated from human gingival explants (7) were cultured in αMEM (Stemcell Technologies, Vancouver), supplemented with antibiotics (penicillin G (Sigma, St. Louis, Missouri) 100 µg/ml, gentamicin (Sigma) 50 µg/ml, amphotericin B (Fungizone, Gibco, Grand Island, N.Y.) 3 µg/ml and 15% fetal bovine serum (Gibco) at 37°C in a humidified atmosphere with 5% CO_2. Cells were removed from their growth surface by a trypsin solution (0.25% trypsin (Gibco) and 0.1% glucose dissolved in citrate-saline (pH 7.8)).

Substrata

Cell lines were typically cultured on tissue culture plastic in either flasks (Corning) or multiwell plates (Falcon, Becton Dickinson, Lincoln Park, N.J.). In some experiments, as noted HGF were cultured on Petri-perm dishes (Hereus) which have a gas-permeable membrane as the floor of the dish which maximizes exposure of cells to gases. In other experiments, titanium (Ti)-coated substrata were used so that the results could be compared with other studies of cell shape and cytoskeletal distribution (8). Smooth or micromachined grooved surfaces were coated with ≈50 nm titanium using a sputter coater. The grooved surfaces had truncated V shaped grooves with a 30 µm pitch (i.e. a 15µm groove and a 15 µm ridge and a depth of 3 µm. These grooved surfaces were used as it had been found that they could alter cell shape and compensate for the disruption of the microtubule system by colcemid (9). Smooth surfaces served as controls. Ti-coated surfaces were cleaned by ultrasonication for twenty minutes in a detergent specifically formulated for tissue culture (7X Cleaning Solution, Flow Laboratories Inc., Mc Lean, VA), copiously rinsed in distilled water, and immediately before use in cell culture, treated for 3 minutes in an argon gas glow-discharge chamber.

Exposure to CH_3SH

The HGF were exposed to CH_3SH according to the procedure and in the apparatus described Ng and Tonzetich (6). In brief, cultures were incubated

simultaneously in humidified sealed chambers perfused with 95% air/5% CO_2. The line leading to the test chamber was equipped with a calibrated permeation tube which emitted a fixed concentration of CH_3SH to the air mixture. The flow rate of gases was 60 ml/min and the gases were directed through teflon lines to 600 ml reservoirs immersed in 37° water to preheat gases prior to their entry into the culture chambers.

Zinc Chloride ($ZnCl_2$) Solutions.

Zinc chloride (Fisher Scientific) stock solutions of pH 5.7 were prepared at a concentration of 15 $X10^{-2}M$, a concentration approximately ten times higher than that of a commercial mouth rinse (which had a concentration of 0.22%). As $ZnCl_2$ solutions have a tendency to form precipitates at higher pHs, the concentration of Zn^{++} in samples was determined by direct flame atomic absorption spectrophotometric analysis (CanTest, Canada). The prepared $ZnCl_2$ solutions were accurate to within ± 10% of the nominal value. For each experiment fresh solutions of $ZnCl_2$ were prepared. Serial dilutions of stock solutions were made to obtain the $ZnCl_2$ concentrations indicated in the various experiments.

Immunofluorescence

Confocal laser scan microscopy (CLSM) of cells stained by using fluorescent probes was carried out as described by Oakley and Brunette (8). In brief, at various times as noted, cells on substrata were rinsed with cytoskeletal stabilizing buffer, fixed 10 minutes in 3.7% formaldehyde (BDH Inc., Toronto, Canada) in phosphate-buffered saline (PBS), and washed in cytoskeletal-stabilizing buffer. To visualize cell morphology, samples were prepared for incubation with fluorescent hydrazides to stain the cell membrane. Following oxidation in 4.2 mM sodium periodate, samples were exposed to fluorescein-5-thiosemicarbazide (Molecular Probes Inc., Eugene, Oregon). Subsequently, preparation for staining of cytoskeletal elements was continued by permeabilization in 0.5% Triton X-100 (Sigma, St. Louis MO), quenching with 0.05% sodium borohydride (BDH Inc.), blocking with 1% bovine serum albumin (BSA, Sigma) and exposure to one or more of the following antibodies or fluorescent stains: Bodipy or fluorescein phallacidin (Molecular Probes Inc.), monoclonal anti beta-tubulin or monoclonal antivinculin (Boehringer-Mannheim GmbH, Mannheim, Germany), sheep

anti-mouse IgG conjugated to Texas Red (Molecular Probes Inc.) or fluorescein isothiocyanate (FITC; Boehringer-Mannheim GmbH). All antibodies were diluted with PBS containing 0.1% BSA.

Microscopy and Morphometrics

Cells stained with the hydrazide were examined through a microscope equipped with epifluorescent illumination (CLSM), Zeiss, Germany) and photographed (Olympus PM4T camera, Kodak Tmax 400). Fields were selected randomly and the only criterion for cell inclusion in the sample was the absence of contact of a cell with other cells. Subsequently, images of the photographed cells were projected and cell outlines as well as corresponding groove-ridge patterns, where applicable, were traced onto paper. The tracings were retraced on the digitizing tablet of a morphometrics system (Videoplan, Zeiss) which calculated cell area, maximum diameter and a form factor (Form Ell), that is a ratio of the minor to major axes. The longest diameter between two edges within the cell was considered the major axis and the minor axis was considered the longest axis perpendicular to the major axis. Form ELL value of 1.0 corresponds to a circular shape and values less than one indicate elliptical or spindle shapes. The angle formed by the major axis to either the grooves or, for smooth surfaces, a line with randomly-selected orientation was termed the orientation angle. For measurement of cell orientation, no attempt was made to distinguish cell polarity and therefore the maximum angle possible was 90°, with a value of 0° indicating a cell that was perfectly aligned with the direction of the grooves. The number of cells used in the estimates of area, D max, form factor ELL and orientation angle ranged between 44 and 145 cells.

Cytoskeletal elements were examined with both epifluorescence and confocal microscopy using a 63x oil-immersion plan-apochromat objective, NA 1.4 (Zeiss) on the CLSM (Zeiss). An argon laser (λ max=488 nm) was used for Bodipy and FITC-labelled cells in confocal microscopy; a helium-neon laser (λ max=543 nm) was used for Texas-Red labels. For double-labelled specimens, the FITC signal was examined first, before the Texas-Red signal. Digital grey-scale images obtained by the CLSM were printed using a video printer (UP-5000, Sony Canada, Richmond, British Columbia).

Protein Synthesis

Confluent HGF cultures were trypsinized, pooled, and 3.5 $X10^5$ cells plated in 60 mm tissue culture plastic dishes and incubated overnight in 2 ml Dulbecco's Minimal Essential Medium (DMEM)(Gibco,) supplemented with 10% FCS. After attachment the medium was discarded and the cultures rinsed three times with PBS then made quiescent during 48 h of incubation in RPMI 1640 (Gibco) medium, devoid of serum. Placing the cells into quiescence was done so that the results could be interpreted as effects on protein synthesis and not as indirect effects produced by alterations of cell proliferation. The RPMI medium was then decanted, the cultures washed three times with phosphate buffered saline, then incubated for 24 hours in proline deficient DMEM which was then supplemented with L-[^{14}C]-proline(250 mCi /mmole, ICN,1.5 µCi per 1.5 ml medium per dish) either in the presence or absence of 10% FCS. In experiments using Zn^{++} the medium was pre-filtered after the addition of $ZnCl_2$ since a precipitate sometimes formed, but, as noted above, Zn^{++} concentrations as determined by flame atomic adsorption spectroscopy were accurate to within 10% of the nominal concentrations. At the end of the labelling period, two protease inhibitors, phenylmethylsulfonyl fluoride and 4-chloromercuricbenzoic acid (Sigma, 50 µg/ml final concentration), were added to the medium and the medium was collected. In cultures in which FCS was absent, FCS was added to the medium to serve as a carrier protein. The medium was then precipitated by the addition of 10% trichloroacetic acid /1% tannic acid, and centrifuged (Eppendorf , Model 5142) for 30 minutes at 4°C. Precipitates were washed three times with ice-cold TCA. The [^{14}-C] radioactivity in the resulting pellets was determined by liquid scintillation counting (Philips PW 4700) and results expressed as the total count of material synthesized and secreted by HGF during the 24 hours of incubation.

Results

Cell Shape

The shapes of control HGF cultured on Petriperm dishes as well as cells exposed to CH_3SH are illustrated in Fig. 1. Cells exposed to CH_3SH were found to be rounder, less spread, and less elongated than unexposed cells. Morphometric analysis of these effects in terms of area, maximum diameter

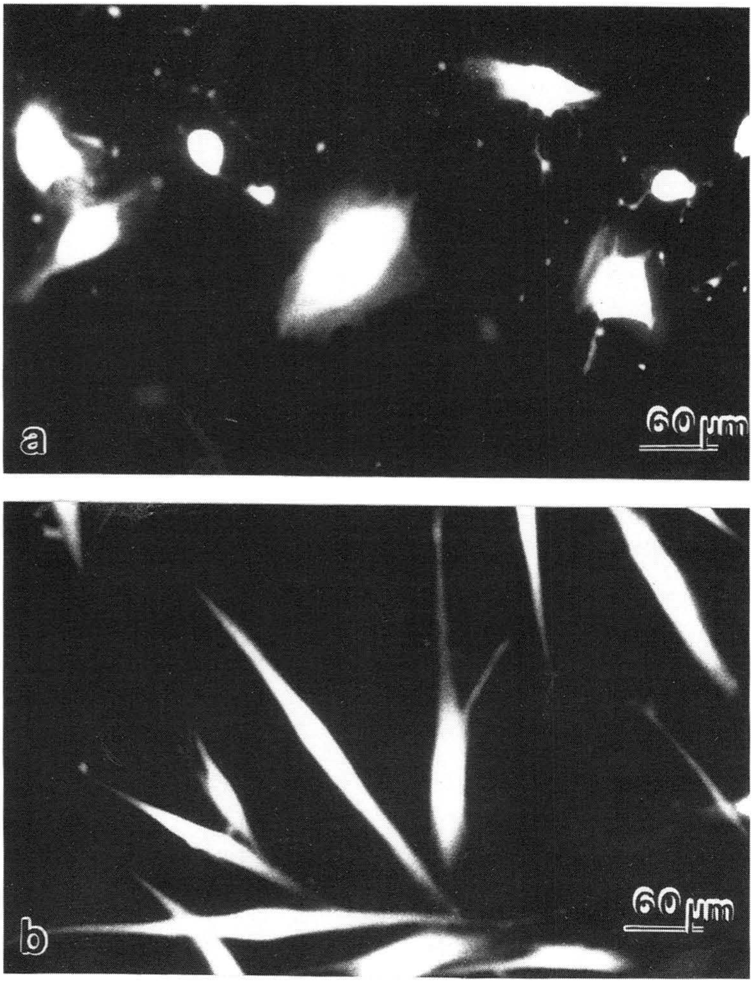


Fig. 1: HGF cultures exposed to CH_3SH (a) or not (b).

(Dmax, an indication of cell elongation) and FORM ELL is given in Table 1 for cells cultured on Petriperm as well as cells cultured on a smooth Ti surface. CH_3SH produced statistically significant effects for some of these parameters, as noted in in Table 1 on both Petriperm and Ti surfaces ($p < .05$, t-test). In every surface tested cell area and Dmax were decreased, but FORM ELL increased by exposure to CH_3SH. As cell shape was altered markedly by CH_3SH it was decided to examine the cytoskeleton.

Table 1: Effects of methyl mercaptan on cell morphology

Surface	Treatment	Area ± SD	Dmax ± SD	Form Ell ± SD N	N
Petriperm	SH	0.91 ± .56	1.29 ± .36*	.41 ± .18*	141
	Control	1.02 ± .78	1.91 ± .71	.18 ± .12	148
TI-Smooth	SH	1.14 ± .77*	1.65 ± .41	.44 ± .15*	29
	Control	1.50 ± .66	1.83 ± .72	.25 ± .16	45
TI-Grooved	SH	1.19 ± .50	2.0 ± .55	.19 ± .07	24
	Control	1.2 ± 1.15	2.17 ± .74	.15 ± .13	25

** Statistically different from control culture on the same surface*

Cytoskeleton

HGF cytoskeleton was clearly affected by exposure of the cells to CH_3SH. Actin microfilament bundles (AFB) were less evident in the exposed cells and their distribution was altered. Whereas in control cells the AFB were distributed uniformly over the breadth of the cell, the AFB in exposed cells were found predominantly in the margins of the cell (Fig. 2). Microtubules (MT) exhibited the opposite distributional behaviour in response to CH_3SH. That is, in unexposed cells the MT were uniformly distributed but in the CH_3SH exposed cells the MT were found in the central portion of the cell (Fig. 3).

Previous work in this laboratory has demonstrated that a grooved surface topography can compensate for the loss of MT which has been produced by treatment with colcemid, a phenomenon named "topographic compensation". It was of interest to determine whether cells cultured on grooved Ti surfaces had a similar response to CH_3SH or whether the topography could compensate for the shape altering effects of CH_3SH. The effects of CH_3SH on cell morphology were very small and non significant on the grooved surface although they tended to be in the same direction (Table 1). Similar effects on the cytoskeleton, however, were observed in the cells cultured on grooved substrata as were observed on smooth substrata. That is, the AFBs which tended to be uniformly distributed in unexposed HGF appeared to decrease in number and were found more marginally on CH_3SH exposed cells (Fig. 4). Once again the MT, which were uniformly distributed in untreated cells, were found more centrally in CH_3SH-exposed cells (Fig. 5). Although the distribution of AFB and MTs and their amount were altered, their orientation was not, and the cells were able to be oriented and spread out on grooved surfaces. Thus, to some extent, the grooved surface was able to compensate for the effects caused by CH_3SH.

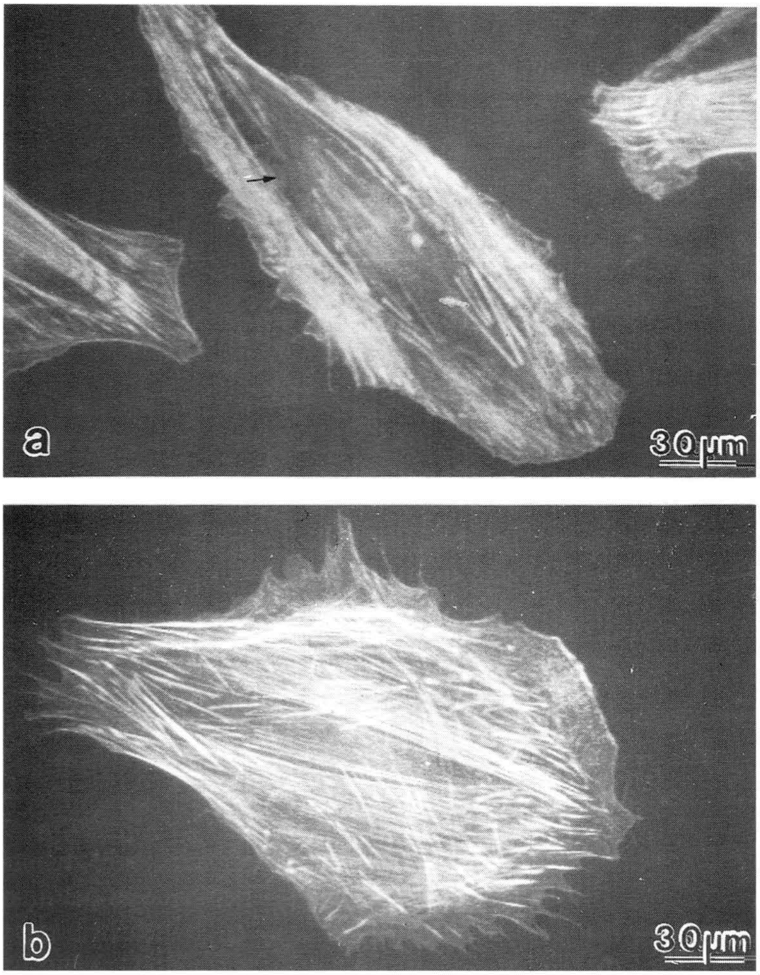


Fig. 2: Actin microfilament bundles are uniformly distributed in control cells (b) while they are predominantly in the margins of the cells when exposed to CH_3SH (a).

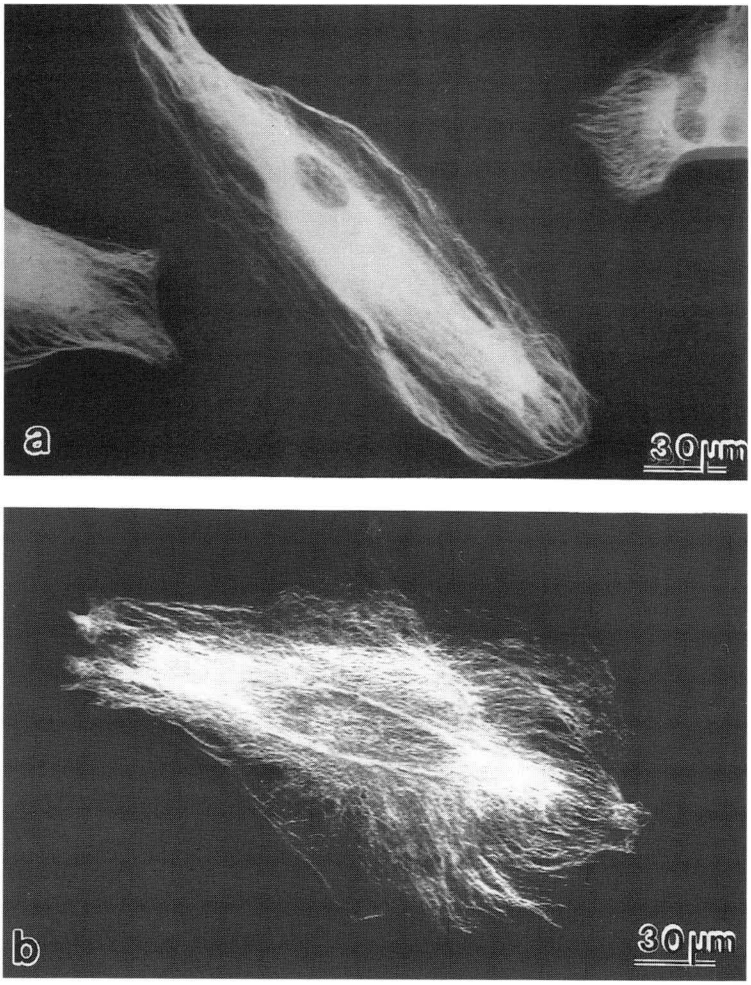


Fig. 3: Microtubules are found in the central portion of the cells exposed to CH_3SH (a) while they are uniformly distributed in the controls (b).

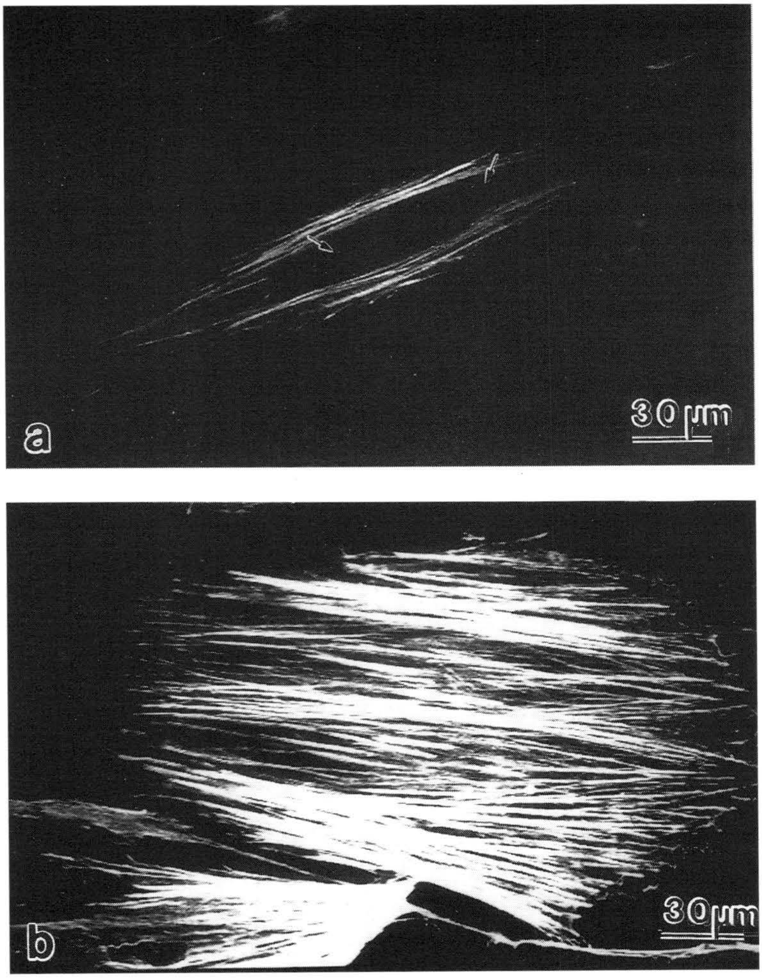


Fig. 4: The actin microfilament bundles are decreased in number and marginalized in human gingival fibroblasts exposed to CH_3SH (a) and evenly distributed in controls (b).

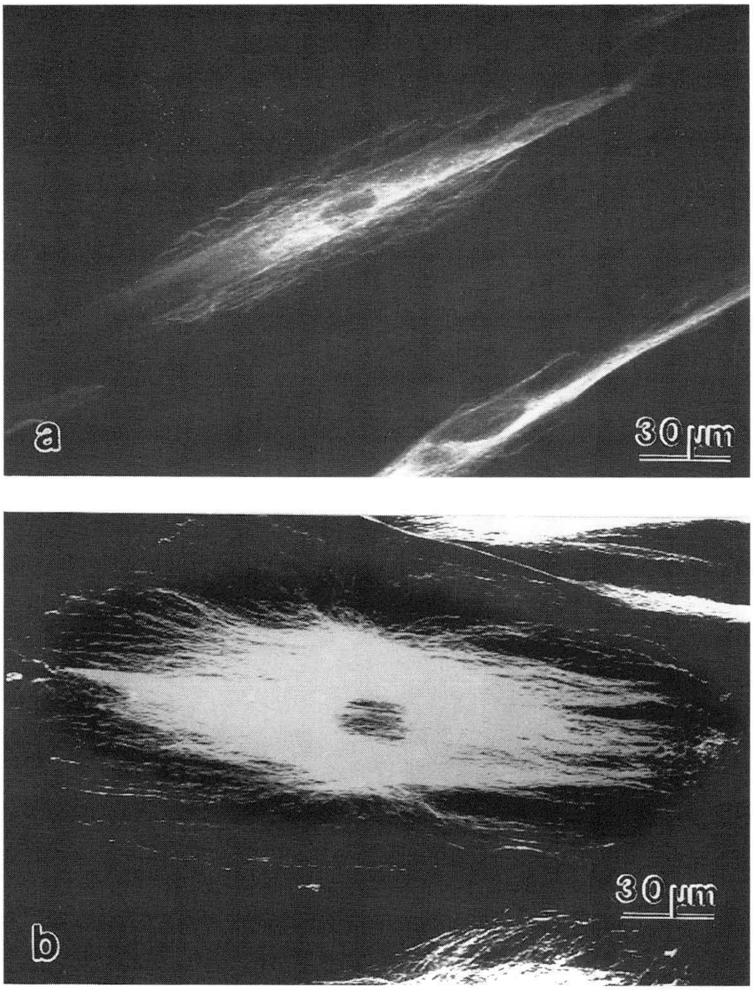


Fig. 5: The microtubules are found more centrally in CH_3SH exposed cells (a) and evenly distributed in controls (b).

Cell Proliferation and Migration

Although not reported here in detail, it was also evident that CH_3SH altered cell proliferation and migration. An example of the morphological effects on growth and migration is shown in Fig. 6. In this experiment equal numbers of cells were plated in small drops on Petriperm dishes and allowed to attach; at any time after plating the size of the colonies is proportional to the rate of cell proliferation and migration as the cells migrate under the influence of

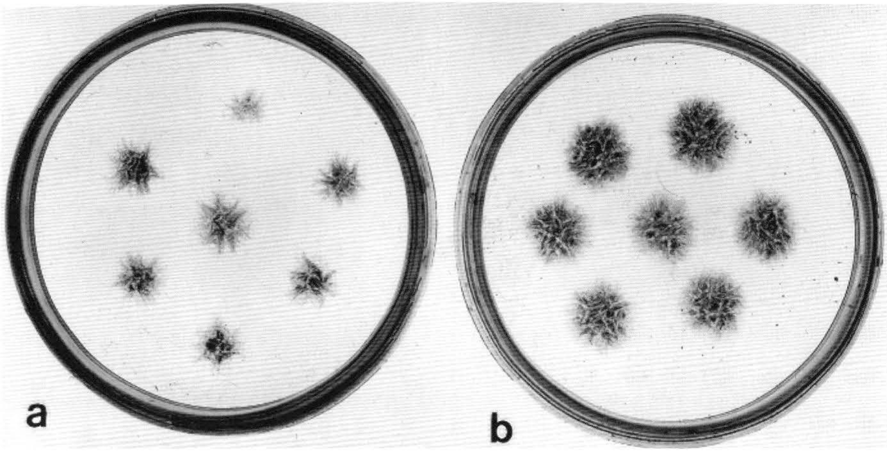


Fig. 6: The same number of cells was incubated on both plates, one in the presence of CH_3SH (a) while the other plate served as a control.

contact inhibition of movement outwards from their initial position. One set of plates was incubated in the presence of CH_3SH while the others served as a control. The colonies formed under control conditions were much larger and more cellular than those formed when the cells were exposed to CH_3SH indicating that CH_3SH inhibited both cell proliferation and migration. The results were confirmed by assessing DNA synthesis in a separate experiment using ^{125}IUDR incorporation, which demonstrated a 38% reduction, and will be reported in more detail as part of another study.

Effect of Zn^{++} on CH_3SH Inhibition of Protein Synthesis.

The ability of CH_3SH to inhibit protein synthesis and the effects of Zn^{++} on this inhibition are shown in Table 2. Methyl mercaptan produced a statistically significant ($p < .05$) 24% inhibition of protein synthesis but this inhibition was blocked by the presence of either 10^{-5} M or $5X10^{-4}$ M Zn^{++}.

Table 2: Effect of CH_3SH and Zn^{++} on Protein Synthesis

Treatment	Medium (dpm x 104)	Cells (dpm x 104)	Total (dpm x 104	Percent Reduction
AIR/CO_2 (Control)	5.16	8.25	13.41 ± .22	——
CH3SH	3.71	6.49	10.2 ± 2.33	24*
CH_3SH^+ 10^{-5}M Zn^{++}	4.17	8.41	12.58 ± 0.4	6
CH_3SH^+ $.5x10^{-4}$M Zn^{++}	4.42	8.45	12.87 ± 1.18	4

** Statistically different from control (p < .05)*

Discussion

Although the presence of VSC, such as CH_3SH, in mouth air is generally considered from the standpoint of its adverse effect on human social interactions, there is gathering evidence that such compounds can have potent biological effects. For example previous studies by Tonzetich and coworkers established that VSC exerts an inhibitory effect in the metabolism of cultured HGF. Exposure of HGF to VSC results in a reduction in total protein and collagen (10), DNA synthesis (11) and proline transport (12). Methyl mercaptan (CH_3SH) has been found to be a stronger inhibitor than H_2S (10). This study has found that CH_3SH also markedly alters cell shape and the cytoskeleton.

There is abundant evidence that cell shape can regulate cell growth (13), gene expression (14), cytoskeleton expression (15), collagenase and stromelysin gene expression (16), ECM metabolism (17), fibronectin (18) and differentiation (19, 20). Indeed. in the view of Ben Ze'ev (21), the concept that the architectural features of eukaryotic cells, which determine cell shape and contacts, can control cellular functions is becoming a central paradigm of cell biology. The actual mechanisms linking cell shape changes to gene activity are still unclear. Ingber (22) has explored a model, "cellular tensegrity", which seeks to link the control of cell and nuclear shape and function to alterations in the cytoskeletal force balance. Ingber has speculated that structural connections between the cytoskeleton and nuclear matrix could lead to alterations in nucleic acid metabolism. In this study it was clear that

the cytoskeleton was altered in cells exposed to CH_3SH in both amount and distribution. It would be expected that such disruptions would have effects on cell function, and indeed protein synthesis and secretion into the medium was found to be inhibited.

Total protein secretion, however, is a rather crude measure of cell function, and other more specific effects would also be suspected to occur. For example, as the microtubule system is involved in the intracellular transport of vesicles and protein particles and CH_3SH exposed cells did not have microtubule tracks running to many regions of the cell membrane, it might be expected that the distribution of membrane proteins might be altered in exposed cells on account of vesicle traffic disruptions. Johnson (personal communication, 1995) is in fact exploring integrin (an integral membrane receptor glycoprotein) distribution in cells exposed to VSC. One of the approaches to the control of oral malodour has been to convert VSC to a non-odorous form, for as noted by Tonzetich (1), in order for a compound to be odorous it must be volatile. Niles and Gafar (23) have used the volatile acid conversion approach using zinc or magnesium salts or sodium bicarbonate. Zn^{++} has also been used in commercial mouthrinses and Yaegaki (24) considers it to be one of the most effective deodorants for oral malodour in orally healthy populations. The reduction of oral malodour, however, does not necessarily imply a reduction in other biological effects, and it was of interest to test Zn^{++} for its ability to inhibit the effects of CH_3SH. In this study Zn^{++} was able to block the inhibition of protein synthesis and secretion by CH_3SH. But, although Zn++ is an essential trace element and is generally considered to be innocuous relative to other divalent cations, Zn^{++} can also produce numerous effects by itself. For example with respect to the HGF used in this study, Ouyang (25) found that, depending on concentration and duration of exposure, Zn^{++} could inhibit cell attachment, proliferation and lead to give rise to cytopathologic alterations in ultrastructure. It is thus clear that studies should be undertaken exploring the effects of differing concentrations and exposures of Zn^{++} to cells with a view to optimizing the use of Zn^{++} in controlling oral malodour as well as the biological effects of VSC.

In summary, this study has shown that CH_3SH can markedly alter cell shape and cytoskeletal element amounts and distributions. These alterations would be expected to alter various cell functions, and indeed overall protein synthesis and secretion was inhibited by CH_3SH. The inhibition of protein synthesis and secretion by CH_3SH could be blocked by Zn^{++}, but additional studies are required to characterize the effects of CH_3SH and other VSC on biological processes and to find whether these effects can be modulated or

reversed by various control strategies such as the use of Zn++ in optimal concentrations.

Acknowledgements

The authors thank Ms. Lesley Weston and Mr. Andre Wong for technical assistance and Medical Research Council of Canada (Operating Grant MT7617) for financial support.

References

1. Tonzetich J. (1977) Production and origin of oral malodor: a review of mechanisms and methods of analysis. *J Periodontol* 48:13-20.
2. Rizzo A.A. (1967) The possible role of hydrogen sulfide in periodontal disease.1. Hydrogen sulfide production in periodontal pockets. *J Periodontol* 5:233-236.
3. Tonzetich J. and Spouge J.D. (1979) Effect of periodontal therapy on volatile sulphur content of mouth air. *J Dent Res* 58:175 (abstract).
4. Yaegaki K. and Sanada K. (1992) Volatile sulphur compounds in mouth air from clinically healthy subjects and patients with periodontal disease. *J Periodont Res* 27:233-238.
5. Tonzetich J. and McBride B.C. (1981) Characterization of volatile sulphur production by pathogenic and nonpathgenic strains of oral Bacteroides. *Archs oral Biol* 26:963-969.
6. Ng W. and Tonzetich J. (1984) Effect of hydrogen suifide and methyl mercaptan on the permeability of oral mucosa. *J Dent Res* 63:994-997.
7. Brunette D.M., Melcher A.H. and Moe H.K. (1976) Culture and origins of epithelium-like and fibroblast-like cells from porcine periodontal ligament explants and cell suspensions. *Archs oral Biol* 21:393-400.
8. Oakley C. and Brunette D.M. (1993) The sequence of alignment of microtubules, focal contacts, and actin filaments in fibroblasts spreading on smooth and grooved titanium substrata. *J Cell Sci* 106:343-354.
9. Oakley C. and Brunette D.M. (1995) Topographic compensation: guidance and directed locomotion of fibroblasts on grooved micromachined substrata in the absence of microtubules. *Cell Motil Cytoskel* 31:45-58.
10. Johnson P.W. and Tonzetich J. (1982) Protein synthesis by human gingival fibroblasts in the presence of thiol compounds. *J Dent Res* 62:275 (abstract).

11. Tonzetich J., Narayanan S. and Page R.C. (1982) Suppression of DNA synthesis of human fibroblasts by hydrogen sulphide. *J Dent Res* 61:260 (abstract).
12. Tonzetich J., Yaegaki K. and Ng W. (1985) Inhibition of proline transport in fibroblast culture by methyl mercaptan. *J Dent Res* 64:306 (abstract).
13. Folkman J. and Moscona A. (1978) Role of cell shape in growth control. *Nature* 273:345-349.
14. Ben-Ze'ev A. (1986) The relationship between cytoplasmic organization, gene expression and morphogenesis. *Trends Biochem Sci* 11:478-481.
15. Ben-Ze'ev A. (1984) Differential control of cytokeratins and vimentin synthesis by cell-cell contact and cell spreading in cultured epithelial cells. *J Cell Biol* 99:1424-1433
16. Werb Z., Tremble P.M., Behrendtsen O., Crowley E. and Damsky C.H. (1989) Signal tranduction through the fibronectin receptor induces collagenase and stromelysin gene expression. *J Cell Biol* 109:877-889.
17. Watt FM (1986). The extracellular matrix and cell shape. *Trends Biochem Sci* 11:482-485
18. Chou L., Firth J.D., Uitto V.J. and Brunette D.M. (1995) Substratum topography alters cell shape and regulates fibronectin MRNA level, MRNA stability, secretion, and assembly in human fibroblasts. *J Cell Sci* 108:1563-1573.
19. Watt F.M. (1987). Influence of cell shape and adhesiveness on stratification and terminal differentiation of human keratinocytes in culture. *J Cell Sci* 8:313-326.
20. Watt F.M., Jordan P.W. and O'Neill C.H. (1988) Cell shape controls terminal differentiation of human epidermal keratinocytes. *Proc Nat Acad Sci USA* 85:5576-5585.
21. Ben-Ze'ev A. (1991) Animal cell shape changes and gene expression. *Biol Essays* 13:207-211.
22. Ingber D.E. (1993) Cellular tensegrity: defining new rules of biological design that govern the cytoskeleton. *J Cell Sci* 104:613-627.
23. Niles H.P. and Gaffar A. (1995) Advances in mouth odor research. In: Rosenberg M. (Ed.) *Bad Breath: research perspectives.* Ramot Publishing, Tel Aviv, pp 55-69.
24. Yaegaki K. (1995) Oral malodor and periodontal disease. In: Rosenberg M. (Ed.) *Bad Breath: research perspectives.* Ramot Publishing, Tel Aviv, pp 87-108.
25. Ouyang Y. (1991) Effect of zinc on the metabolism of thio-treated human gingival fibroblasts. M. Sc. Thesis, The University of British Columbia.

Thiols Modulate Metabolism of Gingival Fibroblasts and Periodontal Ligament Cells

*H. Lancero, J. Niu, and P. W. Johnson**

Volatile sulphur compounds (VSC) are produced in periodontal pockets as a result of the protein breakdown associated with periodontal disease. Previous studies have shown that VSC such as methyl mercaptan inhibit collagen synthesis in fibroblast cell lines (1). In order to explore the mechanism for these effects, we are analyzing changes occurring in total protein, collagens and cell function in cell lines exposed to VSC.

Gingival fibroblasts and periodontal ligament (PDL) cells were exposed to methyl mercaptan for two days in culture. CH_3SH was continuously added at a fixed concentration of 10 ng/ml to the air/CO_2 mixture perfusing the cells using a permeation tube inserted into the gas line leading to test samples. Treated cells were then analyzed for alterations in protein, amounts and types of collagens and cell migration.

Results of slab gel electrophoresis of collagens from fibroblasts indicate that there are changes in procollagen species accompanied by reductions of mature $\alpha1$ and $\alpha2$ type I collagen chains. Identity of procollagens was confirmed by pepsin digestion which also suggested there were reductions in type III collagens. Reverse transcribed polymerase chain reaction (RT-PCR) using primers for type I and type III collagen $\alpha1$ chains indicated a reduction in the ratio of type III to type I collagen mRNA in cells exposed to CH_3SH. PDL cell migration was measured using a "microfence" assay. Results showed that mercaptan inhibited cell migration in this assay.

Results of these studies indicate that methyl mercaptan induces alterations in extracellular matrix molecules with concomitant effects on cell function. These effects are potentially important in the pathogenesis of periodontitis disease and in wound healing.

Introduction

Most pathological and physiological oral malodour arises within the oral cavity

* H. Lancero, J. Niu and P.W. Johnson, Department of Stomatology, Division of Periodontology, Faculty of Dentistry, University of California at San Francisco, C-628, Box 0650, San Francisco, CA 94143-0650, USA.

as a result of volatile compounds which are produced through putrefaction of proteinaceous material by oral microflora. The process involves hydrolysis and putrefaction of protein which occurs normally but is intensified in inflammatory conditions. Inflammation of the periodontium is characterized by a change in microbiota as well as an increase in levels of desquamated host cells and a pooling of blood. Both of these processes increase odour production dramatically by providing large amounts of substrate for proteolytic bacteria (2).

Mouth air is a complex mixture of volatile compounds present in the oral cavity in the parts per billion (ppb) range. Although most bacteria preferentially metabolize carbohydrate, protein becomes an alternative source of energy during periods of abstinence from food and liquid or in areas of stagnation. The main compounds associated with malodour have been shown to demonstrate a positive nitroprusside reaction for thiol groups. Studies have confirmed that the nitroprusside-positive reaction is associated with volatile sulphur compounds (VSC) such as methyl mercaptan and hydrogen sulphide, the two major compounds associated with oral malodour (3). These substances are derived from degraded proteins through dethiolation and demethylation of sulphur-containing amino acids (4). Direct analysis of mouth air samples has demonstrated that hydrogen sulphide, methyl mercaptan and dimethyl disulphide are in the mouth air of all individuals at concentrations of 0.01 to 1 ng/ml and can be elevated by an order of magnitude in patients suffering from periodontitis (3).

VSC and microbiota associated with periodontitis

Periodontitis is characterized by a change in the bacterial population in the gingival sulcus from Gram-positive cocci to motile and non-motile Gram-negative anaerobic rods and spirochetes (5, 6). The Gram-negative organisms are proteolytic and have been implicated as the primary agents responsible for VSC and odour production (7). Early work has shown that gingival crevice scrapings contain several strains of *Porphyromonas* with variable pathogenic potential. The pathogenic strains have been implicated in the etiology of periodontitis (8). They exhibit both proteolytic and collagenolytic activity, have the ability to produce large amounts of VSC, and are decidedly different from non-pathogenic strains in odour-producing capability. The pathogens also metabolize greater amounts of sulphur-containing amino acids and emit up to eight times more total VSC than non-pathogens. In addition, pathogenic species produce large amounts of methyl mercaptan and dimethyl disulphide whereas collagenase-negative strains emit only hydrogen sulphide and limited amounts of mercaptan (9).

Recent work has significantly enlarged the list of oral bacteria which are capable of producing VSC (10). Most of the strains were cultivated from deep periodontal pockets in nine patients with periodontitis. One of the significant aspects of this study is that most of these bacteria were capable of producing high levels of H_2S both in serum cultures and in cultures supplemented with cysteine. Production of mercaptan, however, was restricted to a much smaller number of bacteria. Production of high levels of mercaptan was a characteristic of pathogenic bacteria associated with periodontitis. These included *Treponema denticola, Porphyromonas gingivalis, Porphyromonas endodontalis, Fusobacterium nucleatum, Fusobacterium periodonticum,* and two uncharacterized strains of *Eubacterium* and *Bacteroides.*

Collagens in periodontitis

Inflammation of gingival tissue has a marked influence on the content of collagen within the affected areas and on the species of collagen present. As early as 4 days after the initial accumulation of dental plaque, there is a rapid loss of 60-70% of collagen at the affected site (11). This is characterized by a dramatic reduction in acid soluble as well as insoluble collagen, which is the mature, more cross-linked form of the molecule (12). Whether this phenomenon is a reflection of alterations in collagen synthesis or degradation is a complex question since both mechanisms likely play a role.

Normal human gingiva and periodontal ligament contain approximately 20% type III collagen with most of the remainder composed of type I (13). Experimental characterization of collagens from fibroblasts of normal and diseased gingiva have revealed that inflammed tissues contain only 4% type III collagen. In addition, type I primer, a molecule believed to be associated with abnormal cell metabolism, has been isolated from both inflamed gingival tissue and from fibroblasts cultured from diseased tissue. Since this collagen appears more soluble than the normal type I species, it has been suggested that it may be more susceptible to enzymatic degradation (12).

The rapid loss of connective tissue during inflammation is presumably related to the inordinately high turnover of collagens in periodontal tissue. Radioisotope and biochemical studies have demonstrated that the half life of collagen is one day in the periodontal ligament and five days in the gingiva. This turnover is much more rapid than in tissues such as skin, where the corresponding half life is 15 days (14). Hence, even a small increase in the rate of destruction or a decrease in synthesis would be rapidly reflected in total collagen content of these tissues.

Interactions of methyl mercaptan with cells and proteins

Methyl mercaptan has been examined along with similar thiols for its effects on red blood cells. Studies with CH_3SH along with persulphides (RSSH) have demonstrated that these agents can inactivate a large number of enzyme systems. These include pyruvate kinase and a wide variety of enzymes involved in glycolysis (15). Substantial deficiencies of pyruvate kinase have been associated with certain hyperproliferative red blood cell disorders. Mercaptan synthesis is also elevated in these syndromes and may inactivate pyruvate kinase through interaction with free sulphydryl groups. This appears to be a logical supposition since one aspect of therapy is treatment with thiol reducing agents (16).

Previous studies of saliva incubations with $[^{35}S]H_2S$ have indicated that hydrogen sulphide can incorporate into salivary proteins. Incubations for one and five days demonstrated that all of the H_2S was assimilated by saliva after 24h (17). By one day, electrophoresis and quantitation of radioactivity revealed that the isotope was incorporated into ions, cellular debris and three predominant protein peaks. Although the peaks of protein were not characterized further, they demonstated that the incorporation was specific to certain proteins one of which was not albumin which was identified by gel standards following staining with Coomassie Blue. In additional experiments, insoluble type I collagen and gelatin were also reacted with $[^{35}S]H_2S$ and analyzed by liquid scintillation counting. Both showed strong capabilities to react with H_2S (17).

The experiments described in this manuscript were prompted by associations between oral malodour and periodontal inflammation. Since previous studies had demonstrated interactions between VSC and cells as well as proteins, the experiments were designed to examine the effects of these compounds on both human gingival fibroblasts and periodontal ligament (PDL) cells. Collagens were one of the proteins selected for analysis because of their importance in extracellular matrix (ECM) of the periodontal ligament and their well-characterized changes in periodontitis. Experiments were conducted with methyl mercaptan, the VSC produced in greatest abundance by bacteria associated with severe periodontitis.

Methyl mercaptan alters extracellular matrix of human gingival fibroblasts

Human gingival fibroblasts were cultured in a continuous gas flow system that

allowed a known amount of methyl mercaptan to be added to the 95% air/5% CO_2 gas mixture perfusing the cells. Concentrations of CH_3SH were set at 10 ng/ml in the gas leading to test cultures whereas parallel control cultures received air/CO_2 alone. Experiments were conducted over two day exposures to the gases. Proteins were labelled during the second day of exposure by the addition of [^{14}C]-proline to culture medium.

Initial studies were performed with $5X10^5$ cells plated onto 60mm tissue culture plates followed by precipitation of proteins in 10% trichloroacetic acid (TCA)/1% tannic acid/0.2% fetal bovine serum. Precipitated protein was washed three times with the mixture to remove unbound isotope. The combined activity from medium and cells was then quantitated by liquid scintillation counting. Activity for total protein is illustrated in Table 1. The data show that CH_3SH reduced total precipitable protein in fibroblast cultures. Since these studies were performed with a proline isotope, the results are, however, biased toward the collagens since these proteins contain approximately 25% proline and hydroxyproline, unlike other proteins that do not preferentially incorporate this amino acid.

Table 1: Total and collagenase-digestible proteins of fibroblasts treated with CH_3SH

System	Experiments*		
	Experiment 1	Experiment 2	Experiment 3
Total Protein			
Control	42.270 ± 1303	44.930 ± 2427	33.390 ± 1721
CH_3SH	37.090 ± 1180	40.400 ± 1367	26.890 ± 879
Collagenase-digestible Protein			
Control	12.800 ± 400	7.150 ± 473	16.620 ± 685
CH_3SH	9.640 ± 406	5.740 ± 412	11.230 ± 518

** Data were analyzed by a two-way analysis of variance. Control and CH_3SH-exposed cultures were statistically significant at $p < 0.01$. Values depict means of 5 cultures ± standard deviation.*

To more directly determine alterations of collagens, similar experiments were performed in which aliquots of the same sample were incubated with and without collagenase prior to precipitation. The reduction in activity that could be precipitated by the TCA/tannic acid mixture following collagenase treatment provided an approximate measurement of changes in activity that

could be attributed to the collagens. As indicated in the lower portion of Table 1, collagenous proteins accounted for appreciable portions of the precipitable protein and also demonstrated significant decreases in incorporation of isotope following exposure to mercaptan. The results of these studies prompted further investigations into the effects of CH_3SH on collagen processing.

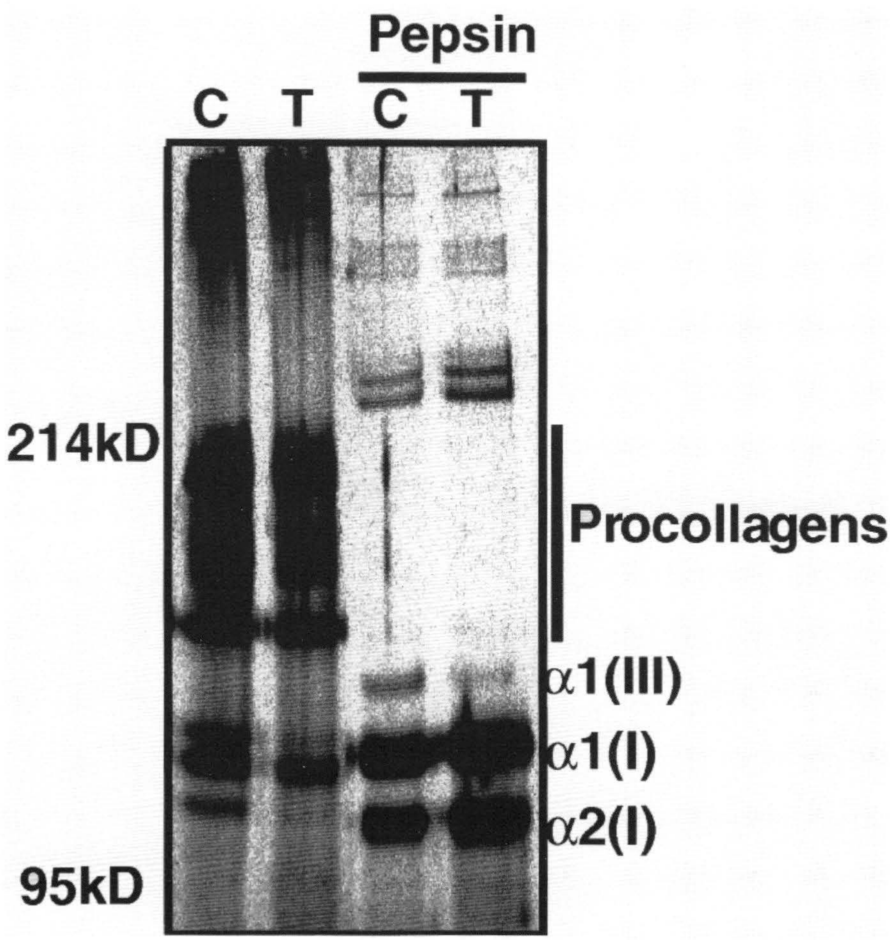


Fig. 1: Modulation of collagen processing by methyl mercaptan. Collagens from Control (C) and Test (T) fibroblast cultures were identified by SDS polyacrylamide gel electrophoresis. Medium was examined prior to and following pepsin digestion.

Effects of methyl mercaptan on metabolism of collagenous proteins were investigated using interrupted SDS slab gel electrophoresis. Medium from [^{14}C]proline-labelled cultures was electrophoresed into 7.5% polyacrylamide gels, the current was interrupted and proteins were reduced by adding 20% (v/v) 2-mercaptoethanol to the wells for 0.5 h. After replacing the reservoir buffer, samples were then run overnight to separate high molecular weight proteins. Protein bands were detected by fluorography. The results of these experiments are shown in Fig. 1.

Control (C) and test (T) samples exposed to CH_3SH for two days are shown in the first two lanes. The second two lanes illustrate results obtained following digestion of these same samples with pepsin. Pepsin removes procollagen extensions on the amino- and carboxy- terminals of the collagen a chains. The second two lanes therefore depict bands that can be attributed either to the $\alpha1$ and $\alpha2$ chains of type I collagen or to the $\alpha1$ chains of type III collagen. This procedure identifies the bands corresponding to the mature collagen a chains in the first two lanes. It also shows the total synthesis of type I and III collagens since in the second two lanes all of the procollagens are converted to collagen and only the mature α chains are present.

Comparison of the first two control (C) and test (T) lanes in figure 1 indicates that minor changes in procollagen banding profiles are present but that the most significant change is a reduction in the mature $\alpha1$ and $\alpha2$ chains of type I collagens in fibroblasts exposed to methyl mercaptan. The identity of these bands is confirmed by comparison with the second two lanes in which only the bands corresponding to mature collagens are present following pepsin digestion. In samples that have been subjected to pepsin digestion, there is also a decrease in intensity of the band corresponding to the mature type III collagen after exposure to CH_3SH. These findings indicate that methyl mercaptan may inhibit processing of procollagens to collagen and may, in addition, inhibit total synthesis of type III collagen.

The effects against type III collagen were further investigated using RT-PCR. The mRNA from test and control fibroblasts was reverse transcribed into cDNA using reverse transcriptase. Relative expression of type I and type III procollagen mRNA was then investigated in a PCR using primers that were derived from the cDNA sequence for type I and type III procollagens. Primers were designed to give a product at 387bp for type III procollagen and 218bp for type I procollagen. Products were labelled by running the PCR in [α-^{32}P]-dCTP and were detected using 5% polyacrylamide slab gels and autoradiography.

As shown in Fig. 2, the control fibroblasts exhibit both bands in increasing intensity as samples are taken from successive cycles of the same PCR. The

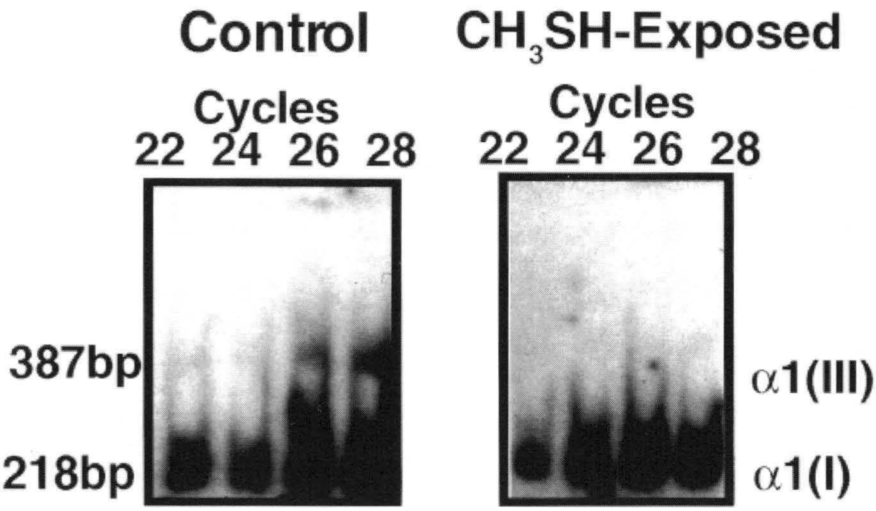


Fig. 2: Reverse transcribed polymerase chain reaction of mRNA from control and test fibroblasts exposed to methyl mercaptan. Synthesized cDNA was probed using primers specific for type I and type III collagen. Bands corresponding to these products are indicated.

fibroblasts exposed to methyl mercaptan exhibit only the band corresponding to the type I procollagen but not the type III in similar cycles. Since the band for the type I procollagen is of similar intensity in both gels, the results support the interpretation that the reason for reduced synthesis of type III procollagens in cells exposed to CH_3SH is related to decreased transcription of mRNA.

An aspect of these experiments that should be stressed is that periodontitis is characterized by a rapid loss of type I and type III collagens but a preferential loss of type III. Inhibition of conversion of type I procollagens to mature collagen would likely result in an abnormal extracellular matrix that would be more susceptible to degradation. The reduction of type III collagen synthesis in cultures exposed to methyl mercaptan is also in accordance with studies that have documented a preferential decrease in type III collagen in inflamed periodontal tissues (12).

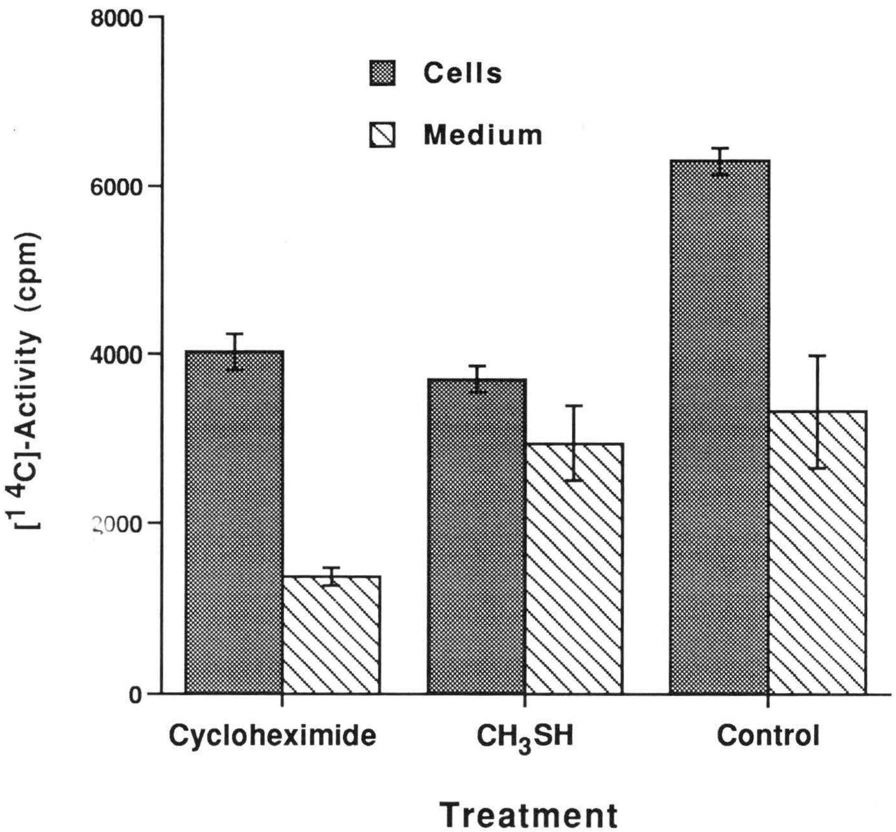


Fig. 3: Protein analyses of PDL cells treated with methyl mercaptan and cycloheximide. Labelled proteins were precipitated with TCA/tannic acid and quantitated by liquid scintillation counting. Results are from triplicate cultures ± standard error.

Methyl mercaptan alters extracellular matrix of human periodontal ligament cells

Studies with human periodontal ligament (PDL) cells exposed to methyl mercaptan were also performed. In experiments analogous to those described in Table 1, analyses for total protein were conducted by labelling proteins with [^{14}C]-proline and precipitating with TCA/tannic acid. PDL cells exposed to CH_3SH for two days exhibited reductions in protein predominantly in the medium fraction (Fig. 3). As a comparison, control PDL cells exposed to cycloheximide (0.1 mg/ml) during the labelling period were also analysed

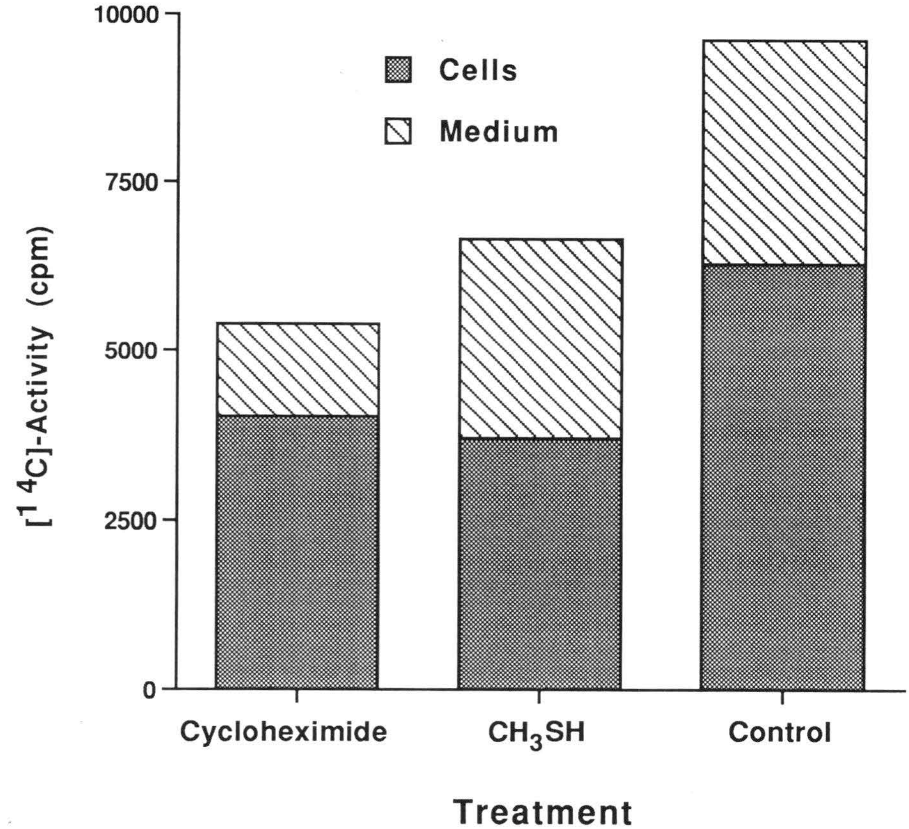


Fig. 4: Total proteins of PDL cells treated with methyl mercaptan and cycloheximide. Total labelled protein from triplicate cultures is shown.

for protein. The results indicate that cells exposed to this inhibitor exhibited reductions in proteins associated with both cells and medium.

In Fig. 4, these results are presented as a combined graph of proteins in medium and cells. Exposure to CH_3SH reduced proteins in combined medium and cell fractions by approximately 30%. However, cycloheximide was more efficient at reducing overall levels of total protein.

As shown in Fig. 5, analysis of procollagens using slab gel electrophoresis was also performed for PDL cells exposed to methyl mercaptan. In test cultures not subjected to pepsin digestion, both the $\alpha1(I)$ and $\alpha2(I)$ bands were reduced when compared with controls. Similarly, the $\alpha(III)$ chains observed following pepsin digestion were reduced in mercaptan-exposed samples. These results and the results from the analyses for total protein indicate that methyl mercaptan has similar effects on gingival fibroblasts and PDL cells.

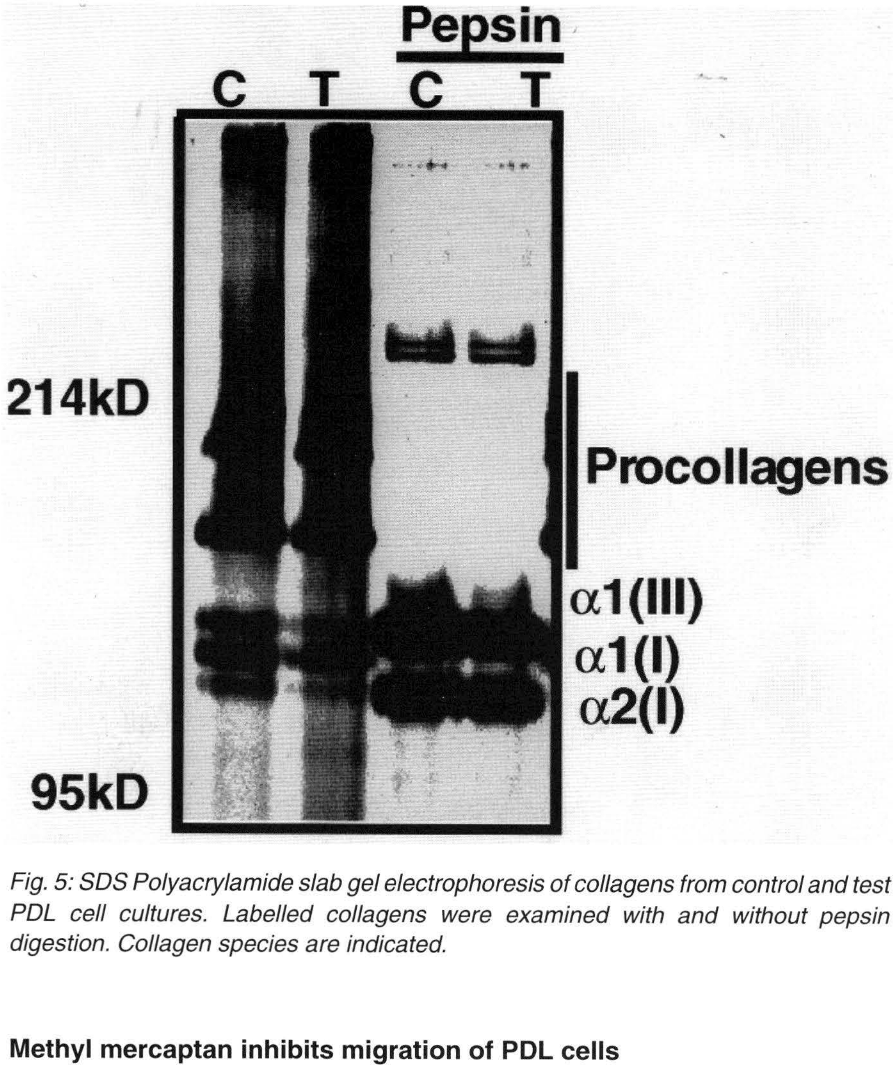


Fig. 5: SDS Polyacrylamide slab gel electrophoresis of collagens from control and test PDL cell cultures. Labelled collagens were examined with and without pepsin digestion. Collagen species are indicated.

Methyl mercaptan inhibits migration of PDL cells

Studies performed with Chinese hamster ovary cells have indicated that protein synthesis inhibitors such as cycloheximide can markedly inhibit migration of some cell types (18). Since cell motility is necessary for proper healing of wounds and would also affect healing following periodontal therapy, the effect of methyl mercaptan on this biologic function is of particular interest. Cell migration was quantitated using a "microfence" assay. Equal numbers of PDL cells (5 X 10^4 per well) were plated through wells of a dot-blot apparatus that sandwiched a "microfence" grid with circular holes onto plates of sterile, tissue culture plastic. All parts of the apparatus had been previously

gas sterilized. Following three hours of attachment, the tissue culture plastic was removed, immobilized to the bottom of tissue culture dishes with sterile vacuum grease and covered with αMEM containing 10% fetal bovine serum. Cells were allowed to recover for an additional 4 hours. Images of the same circles of attached cells were then captured by computer at 0 time, 14h and 24h of migration and areas were quantitated by computer imaging. The area of circles of cells was quantitated in the NIH Image program that was used for capture. Values were expressed as:

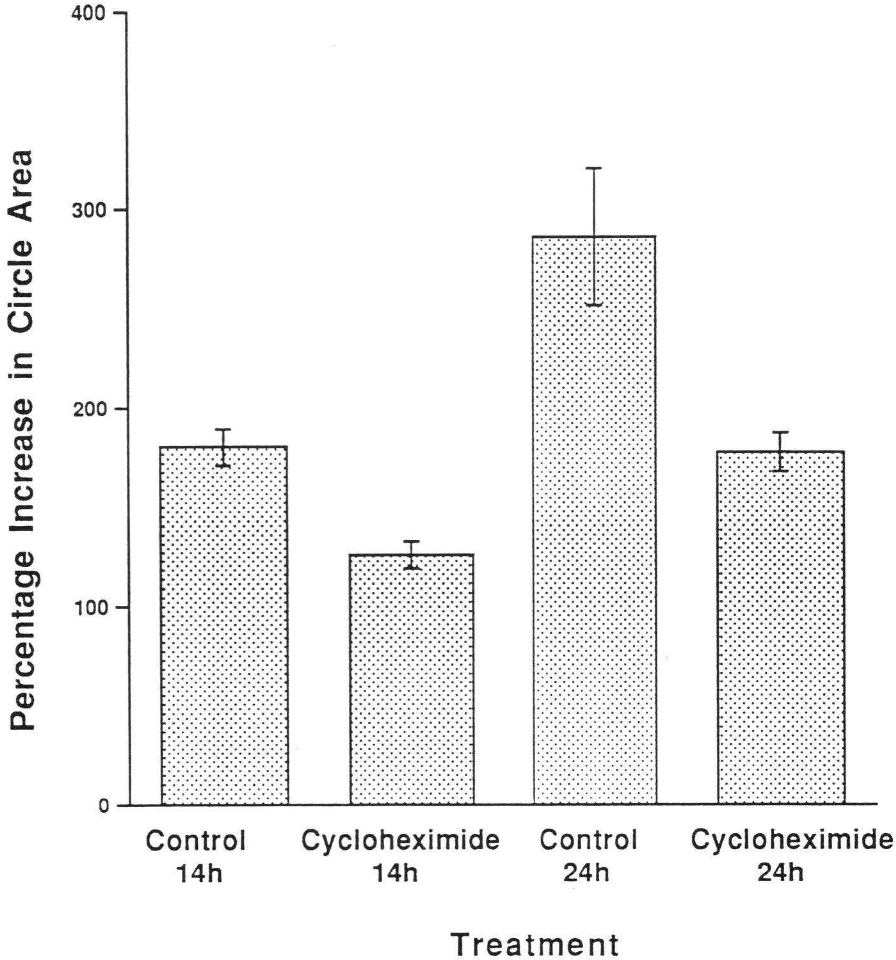


Fig. 6: Migration of cycloheximide-treated PDL cells. "Microfence" assay of PDL cell migration at 14h and 24h time points.

$$\text{Percentage Increase in Circle Area} = 100 \times (\text{Area at 14 or 24h - Area at 0h})/\text{Area at 0h}$$

As shown in Fig. 6, cycloheximide-treated PDL cells tested in this assay exhibit an inhibition of cell migration. This inhibition is apparent at 14h and reaches differences of approximately 40% at 24h. Although some of the difference may be due to changes in cell proliferation, the time of cell division for these cells is approximately every 24h to 36h. Since the assay is complete at 24h, the results can be predominently attributed to differences in migration.

Table 2: Migration of PDL cells exposed to methyl mercaptan

Cells	Experiments*		
	Experiment 1	Experiment 2	Experiment 3
Control-12h	122 ± 13	46 ± 7	68 ± 5
CH_3SH-12h	79 ± 12	19 ± 7	52 ± 8
Control-24h	224 ± 18	135 ± 15	126 ± 6
CH_3SH-24h	146 ± 22	51 ± 18	95 ± 8

** Data depict percentage increase in cell circle area with associated standard error. Values were obtained using 5 circles of each cell type with the same circles captured by computer imaging at 0, 12 and 24 hours. Data were analyzed using the Scheffe's F procedure in a two-way analysis of variance. Migration values for controls compared with CH_3SH were significantly different ($P < .05$) at 24h of migration.*

Similar assays were performed with PDL cells exposed to methyl mercaptan during the migration period. Images of the migrating cells at 0 h and 24 h are shown in Fig. 7. The quantitation of these images is presented in Table 2. Results of three independent experiments demonstrate a significant inhibition of cell migration as shown by this assay.

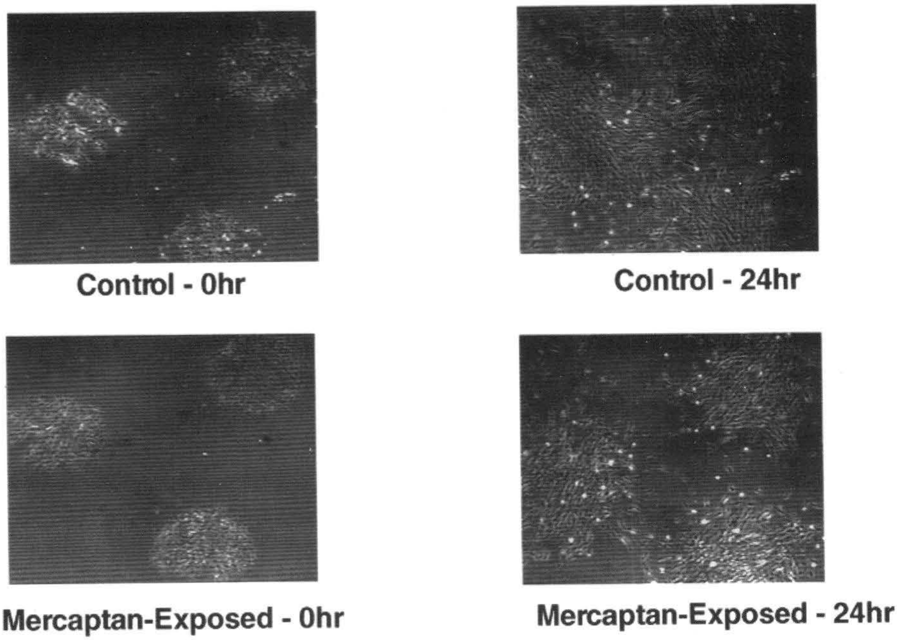


Fig. 7: Images of PDL cell migration as controls or tests exposed to methyl mercaptan. Images are of the same fields at 0h and 24h migration time points.

Concluding remarks

The purpose of this presentation was to document effects of methyl mercaptan on metabolism of total proteins and collagens in both human gingival fibroblasts and periodontal ligament cells. CH_3SH has marked inhibitory effects on both protein synthesis and processing and results in alterations of extracellular matrix proteins. These changes modulate the responses of cells and affect basic biologic processes such as cell migration. The concentration of gas used in these studies (10 ng/ml) was chosen as a rough approximation of VSC levels in periodontal pockets and is well within the range that can be generated by by biologic systems. The results therefore indicate that compounds such as methyl mercaptan may play an important role in the pathogenesis of periodontitis. They may also modify wound healing responses in the oral cavity.

These studies were supported by the NIDR Research Grant in Oral Biology 1 P50 DE10306.

References

1. Johnson P.W., Ng W. and Tonzetich J. (1992) Modulation of human gingival fibroblast cell metabolism by methyl mercaptan. *J Periodont Res* 1992: 27, 476-483.
2. Sulser G.F., Brening K.H. and Fosdick L.S. (1939) Some conditions that affect the odor concentration of the breath. *J Dent Res* 18:355-359.
3. Tonzetich J. (1971) Direct gas chromatographic analysis of sulphur compounds in mouth air in man. *Archs oral Biol* 16:587-597.
4. Tonzetich J., Johnson P.W. and Ng S.K. (1979) Pathways of cystine metabolism in human saliva. *Archs oral Biol* 24:35-39.
5. Löe H., Theilade E. and Jensen S.B. (1965) Experimental gingivitis in man. *J Periodontol* 36:177-187.
6. Dzink J.L., Socransky S.S. and Haffajee A.D. (1988) The predominant cultivable microbiota of active and inactive lesions of destructive periodontal diseases. *J Clin Periodontol* 15:316-323.
7. McNamara T.F., Alexander J.F. and Lee M. (1972) The role of microorganisms in the production of oral malodor. *Oral Surg, Oral Med, Oral Path* 34:41-48.
8. White D. and Mayrand D. (1981) Association of oral *Bacteroides* with gingivitis and adult periodontitis. *J Periodont Res* 16:259-265.
9. Tonzetich J. and McBride B.C. (1981) Characterization of volatile sulphur production by pathogenic and non-pathogenic strains of oral *Bacteroides*. *Archs oral Biol* 26:963-969.
10. Persson S., Edlund M-B., Claesson R. and Carlsson J. (1990) The formation of hydrogen sulphide and methyl mercaptan by oral bacteria. *Oral Microbiol Immunol* 5:195-201.
11. Page R.C. and Schroeder H.E. (1976) Pathogenesis of inflammatory periodontal disease. A summary of current work. *Lab Invest* 34:235-249.
12. Narayanan A.S. and Page R.C. (1976) Biochemical characterization of collagen synthesized by fibroblasts from normal and diseased human gingiva. *J Biol Chem* 251:5464-5471.
13. Butler W.T., Birkedal-Hansen H., Beegle W.F., Taylor R.E. and Chung E. (1975) Proteins of the periodontium. Identification of collagens with the [a1(1)]2a2 and [a1(III)]3 structures in the bovine periodontal ligament. *J Biol Chem* 250:8907-8912.
14. Sodek J. (1977) A comparison of the rates of synthesis and turnover of collagen and non-collagenous proteins in adult rat periodontal tissues and skin using a microassay. *Archs oral Biol* 22:655-665.
15. Valentine W.N., Toohey J.I., Paglia D.E., Nakatani M. and Brockway R.A. (1987) Modification of erythrocyte enzyme activities by

persulphides and methanethiol: possible regulatory role. *Proc Natl Acad Sci USA* 84:1394-1398.

16. Arnold H., Blume K.G., Lohr G.W., Boulard M. and Najean Y. (1974) "Acquired" red cell enzyme defects in hematological diseases. *Clin Chim Acta* 57: 187-189.
17. Tonzetich J. and Lo K.K.C. (1978) Reaction of hydrogen sulphide with proteins associated with the human mouth. *Archs oral Biol* 23:875-883.
18. Rosen E.M., Meromsky L., Setter E., Vinter D.W. and Goldberg I.D. (1990) Quantitation of cytokine-stimulated migration of endothelium and epithelium by a new assay using microcarrier beads. *Exp Cell Res* 186:22-31.

Effect of Volatile Sulphur Compounds on Periodontal Tissues and Cellular Metabolism: an Overview

*J. Tonzetich**

While the importance of volatile sulphur compounds (VSC) as primary components responsible for oral malodour is well established, there is strong evidence that they may also play a significant role in undermining the integrity of periodontal tissues. The objective of this overview is to summarize and interpret the findings derived from various studies how the VSC, especially methyl mercaptan (CH_3SH), may be involved in the pathogenesis of periodontitis and other oral inflammatory conditions.

The results of studies presented by Drs. Brunette, Johnson, Waterfield and Yaegaki in the previous chapters, along with additional findings by R. MacKay and others in my laboratory, collectively provide cogent evidence that H_2S and CH_3SH at concentrations produced by salivary putrefaction exert a number of adverse effects on protein metabolism and other vital biochemical processes in fibroblast cell cultures. Both thiol compounds have been found to alter the turnover rate of collagen which could account for the increased fibrinolysis of gingival, periodontal ligament, and bone collagen observed in periodontitis.

Using confocal scanning laser microscopy, Brunette observed that exposure of human gingival fibroblasts (HGF) to CH_3SH results in a marked alteration in cell shape and distribution of cytoskeleton components. He theorized that these changes may effect cell mobility, gene expression and cell function. This interpretation is supported by Johnson's findings which show that CH_3SH decreases the migration of periodontal ligament fibroblasts. He ascribed this effect to a demonstrated reduction of $\beta 1$ integrin on the surface of exposed cells. The interference in translocation of $\beta 1$ integrin from the interior to the cell surface caused by CH_3SH may be the result of its ability to disturb the cytoskeleton.

Brunette, Johnson and Yaegaki all provided strong evidence that CH_3SH elicits a marked alteration in protein metabolism of HGF. Brunette reported that CH_3SH suppressed DNA synthesis and proline transport and reduced total protein content of the treated cell cultures. In addition to a reduction in proline transport, Yaegaki showed that CH_3SH also reduced collagen

---

* J. Tonzetich, Department of Oral Biology, Faculty of Dentistry, University of British Columbia, 2199 Wesbrook Mall, Vancouver, B.C., Canada V6T 1Z3.

synthesis by about 39% and increased intracellular and extracellular degradation of collagen by approximately 33 and 55%, respectively. This resulted in an overall 70% lower collagen content of the CH_3SH-exposed cultures compared to the controls. It is noteworthy that at the onset of the inflammatory stage of periodontitis there is an initial rapid loss of approximately 70% of collagen of infiltrated gingival tissues. Yaegaki's study suggests that this may be attributed to a combined effect of decreased synthesis and increased rate of destruction which he noted in the HGF cultures.

Slab gel electrophoresis results presented by Johnson demonstrate that exposure of cell cultures to CH_3SH caused reductions in both the mature $\alpha 1$ and $\alpha 2$ chains of type I collagen and type III procollagen. This is in agreement with his polymerase chain reaction observations which indicated that CH_3SH suppressed mRNA synthesis for type III procollagen. These results suggest that CH_3SH effects collagen synthesis through mechanisms involving alteration in procollagen processing and changes in gene expression.

The presentation by Waterfield suggests a major plausible pathway for degradation of collagen in cell cultures exposed to CH_3SH. He showed that through its ability to activate cytokine production, CH_3SH is capable of stimulating the immune response cascade of reactions that can lead to tissue destruction. CH_3SH can exert its effect at a number points of the pathway. To begin with, it can augment the LPS-induced activation of IL-1 production by mononuclear cells. The increased levels of IL-1 stimulate greater production of PGE_2 and cAMP by a variety of target cells, which in turn enhance the production of collagen-degrading neutral proteases. Furthermore, Waterfield showed that CH_3SH alone or in synergism with IL-1, LPS and IL-1/LPS, enhanced PGE_2, cAMP and collagenase production by HGF. Alone, it increased the production of each of the three components by approximately 40% and in synergy with IL-1 and LPS by 70%. These findings imply that CH_3SH produced by the periodontopathic bacteria could contribute to tissue destruction in periodontitis by exerting both a direct effect on fibroblasts and indirectly through mononuclear cells.

It is apparent from the above and other findings that volatile thiol compounds can adversely affect cell metabolism of cell cultures and tissue slices. The question arises, can VSC be produced in sufficient concentrations in the gingival crevice to elicit the described changes in the intact tissues? The answer is yes.

In chapter 3, Coil showed GC profiles which illustrated the presence of VSC (H_2S, CH_3SH, $(CH_3)_2S$ and $(CH_3S)_2$) in the air collected from specific gingival sites. The GC analysis revealed that the types and amounts of individual

VSC components present depends on the depth of pockets and the severity of inflammation. Thus while H_2S was the dominant VSC component found in shallow pockets, the concentrations of CH_3SH and total VSC increased with pocket depth and severity of inflammation. In some cases, CH_3SH was the overwhelmingly dominant component aspired from the latter sites.

Interestingly he found, using monoclonal and polyclonal antibodies against type I collagen, that gingival fluid collected from inflamed active periodontal sites exhibiting higher VSC and CH_3SH levels, contained elevated levels of type I collagen fragments. This supports the thesis that increased rate of collagen turnover may result from higher VSC levels.

The next question that arises is whether VSC can transmigrate from the gingival crevice across the crevicular epithelium and basal membrane to the underlying stroma. The answer again is a definite yes. The functional and crevicular epithelium is generally considered to be the weakest barrier in the mouth as it is constantly exposed to inflammatory agents and mechanical stresses, and is devoid of the surface protective keratin layer. The following properties determine the relative ability of penetrants to transverse the epithelial and basal membrane layers of the mucosa: a) small molecules diffuse most readily, b) molecules diffuse faster than the ions, c) volatile molecules diffuse more readily than the non-volatiles, and d) diffusibility is enhanced if the molecule is soluble in both polar and non-polar solvents. This implies that small volatile molecules such as H_2S and CH_3SH, which are soluble in polar and non-polar solvents, may effectively penetrate the oral mucosa.

Utilizing the above illustrated two-chamber perfusion apparatus (Fig. 1) and radioactive analysis (LSC) of the $[^{35}S]$-H_2S-treated mucosa and PBS effluent, W. Ng and I have demonstrated that during the treatment period H_2S diffused through all three layers (epithelial, basal membrane, connective tissue) of unkeratinized porcine sublingual mucosa and that a significant amount of $[^{35}S]$ label was retained by the tissues. Moreover, it was found that both H_2S and CH_3SH increased the permeability of non-keratinized mucosa to ions $[^{35}S]$-SO_4 and molecules $[^3H]$-prostaglandin (PGE_2) and $[^{14}C]$ and FITC-labelled *Escherichia coli* endotoxin (LPS). The induced increase in permeability was dependent both on the length of exposure and concentration of H_2S and CH_3SH applied to the mucosa. Over a 50% increase in permeability was attained in a relatively short period of 60 to 120 minutes of exposure to an atmosphere consisting of 15 ng of H_2S or CH_3SH per ml of 95% air/5% CO_2.

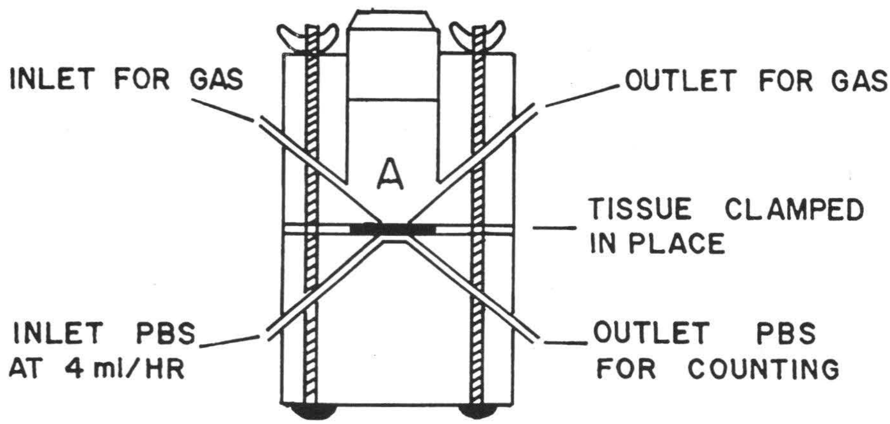


Fig. 1: A modified two-chamber perfusion apparatus. The perfusion units were constructed from Teflon. For each test, a biopsied section of porcine sublingual mucosa was mounted, with the epithelial surface facing upward, over the central aperture (0.0491 cm^2) separating the two chambers held in place by three tightened screws. The VSC and labelled penetrants were applied to the epithelial surface in the upper chamber (A) and perfused through the mucosa into the lower chamber. The inlet-outlet ports in the lower chamber provided a constant flow of phosphate buffered saline (PBS) bathing the connective tissue surface. Aliquots of PBS effluent, which were collected at 20 min intervals over the 3 h duration of the experiment conducted at 37°C, were analysed for radioactivity by liquid scintillation counting.

Table 1: Reversibility of CH_3SH Effect on Permeability of Mucosa to PGE_2

System	% Change in Permeability
Control ≠	- 7.7
CH_3SH-treated §	+ 66.0
$CH_3SH/ZnCl_2$ *	- 2.5

≠ Mucosa was exposed to 95% air/5% CO_2 atmosphere for 60 min.
§ Mucosa was exposed for 60 min to 15 ng CH_3SH/ml 95% air/5% $C0_2$ atmosphere.
** Following 60 min of exposure to 15 ng CH_3SH per ml 95% air/5% $C0_2$ atmosphere, mucosa was treated for 15 min with 0.22% $ZnCl_2$ solution.*

The results in Table 1 merit special interest as prostaglandins can directly suppress synthesis and turnover of collagen and, as already cited, augment the production of endotoxininduced collagenase. The fact that PGE_2 increased the permeability by 66%, strongly implies that the disruption of the permeability barrier by volatile thiols could enhance the accessability of this

inflammatory mediator to the connective tissue.

It should also be noted that the Zn^{++} ion appears to act as a protective agent for maintenance of the permeability barrier. This is consistent with the reported findings that Zn-deficient sulcular epithelial lining is more permeable to proteins than its normal counterpart. Exposing the epithelial surface to $ZnCl_2$, either pre- or post-exposure to CH_3SH restored the permeability of the mucosa to a control state. It is apparent from these and other observations that Zn ion can nullify the adverse effects of volatile thiol compounds on tissues and cellular metabolism.

It is proposed that the Zn^{++} ion exerts its protective effect on the extracellular matrix through the following mechanisms. When the mucosal tissue is exposed to Zn^{++}, the ion readily reacts with tissue anionic carboxyl groups and thus provides a reservoir of Zn^{++} in the treated tissues. In presence of thiols, Zn^{++} ion also readily reacts with mercaptides to form inactive zinc mercaptides. The reservoir of Zn^{++} associated with carboxyl can be released and made available to react with thiol groups if present. Furthermore, proteoglycans (PG) in an aggregated state behave as a molecular sieve in regulating tissue permeability. As PG are aggregated through disulphide linkages, and as these bonds are susceptible to cleavage by thiol compounds, it is highly probable that these bonds are cleaved by thiols such as CH_3SH. This would result in deaggregation of PG and an increase in permeability. The implications of these findings are of major importance as they suggest that Zn^{++} ion may be an useful therapeutic agent for treatment and prevention of VSC-exacerbated conditions.

While it is well established that bacterial endotoxins are the primary etiologic agents in initiating destruction of periodontal tissues, there is considerable controversy as to whether they can diffuse through intact healthy tissues. Since we have observed that VSC can markedly increase permeability of oral mucosa to $[^{35}S]$-SO_4^{2-} and $[^{3}H]$-PGE_2 penetrants, an investigation was undertaken to determine whether CH_3SH can also enhance the permeability of *Escherichia coli* endotoxin.

The experiments depicted in Table 2 employing $[^{14}C]$-LPS indicate that although LPS can diffuse through all three tissue layers, there is a large variation in its capacity to diffuse through different specimens. Whereas it readily diffused through three specimens, it abruptly decreased or stopped in three others after the initial 20 minutes of the experiment, and in one instance it failed to emerge in the PBS effluent. These results provide an explanation for the controversy as to whether normal unkeratinized oral mucosa is permeable to LPS. While the condition of the tested specimens may be an important factor, the variability in thickness of specimens as well as

differences in types and concentrations of LPS preparations used in different investigations may also add to the inconsistency of reported data. It is difficult to obtain tissues of uniform thickness even from a single tissue source. The problem is further compounded if the specimens are compared from different sources.

Table 2: Variability in permeability of oral mucosa to [^{14}C]-LPS

Fraction	dpm [^{14}C]-activity of each effluent fraction						
Number	T-1	T-2	T-3	T-4	T-5	T-6	T-7
1	665	653	263	173	122	44	0
2	109	193	54	6	4	0	0
3	109	143	18	2	0	0	0
4	69	62	20	4	0	0	0
5	139	40	21	0	0	0	0
6	156	35	10	0	0	0	0
7	32	24	1	0	0	0	0
8	56	27	807	0	0	0	0
9	5	117	22	0	0	0	0

Table 2 illustrates the diffusion of [^{14}C]-LPS through 7 different mucosal tissues (T-1 to T-7). For the study, 100 μl of [^{14}C]-LPS solution (2 mg [^{14}C]-LPS/ml PBS) containing 50,000 dpm ^{14}C-activity was applied to each tissue. Effluent fractions of 1.0 ml of PBS were collected and assayed for ^{14}C-activity by LSC.

Even though the three mucosal specimens exhibited good initial permeability to LPS, their permeability was markedly decreased over the 3-hour duration of the experiment. The pretreatment of mucosa with CH_3SH increased its permeability to LPS by 2 to 3-fold and modified the pattern of [^{14}C]-LPS diffusion from that of the control systems (Table 3).

Whereas the diffusion was markedly decreased with time in controls (Table 2), it remained comparatively constant during the same period in the CH_3SH-exposed systems. It appears that LPS is deposited on the surface of the epithelial tissue thus causing diminution with time in the permeability. It is conjectured that the LPS coat on the surface impedes the passages of remaining LPS in solution.

Table 3: Increase in diffusion of [^{14}C]-LPS through mucosa pretreated with methyl mercaptan

Fraction Number	Average dpm [^{14}C]-LPS Activity/Fraction* Unit 1 Test	Unit 1 Control	Unit 2 Test	Unit 2 Control
1	34	9	110	20
2	76	26	98	38
3	19	17	98	54
4	32	18	83	46
5	48	18	60	28
6	95	9	51	54
7	50	10	64	28
8	30	4	53	53
9	8	0	36	20
TOTAL	392	111	653	341

* Each value represents an average of 3 separate experiments.
Tissues were subjected to a flow rate of 6.5 ml/min for 3 h of either 95% air/5% CO_2 (control), or air/CO_2 admixed with 10 ng CH_3SH/ml air/CO_2 (test) atmosphere. They were then overlayed with 100 µl of ^{14}C-LPS and evaluated for a period of 3h according to conditions described for Table 2.

This interpretation is supported by cytofluorescent analysis of the control and CH_3SH treated specimens in which FITC-labelled LPS was used as penetrant. It revealed intense fluorescent staining on the superficial surface of the epithelial layer of control sections. In contrast, the specimens that were exposed for three hours to 15 ng CH_3SH per ml 95% air/5% CO_2 atmosphere yielded a more intense and deeper zone of fluorescent labelling of the surface epithelial layer, and in addition, a discernible distribution of fluorescence throughout the mucosa. The comparison of fluorescence intensity of control and CH_3SH-treated systems clearly showed that CH_3SH had enhanced the penetration of the FITC-labelled endotoxin.

Microscopic and biochemical studies have been performed using light, scanning (SEM), and transmission electron microscopy (TEM), SDS/PAGE slab gel electrophoresis, and immunofluorescence techniques, to determine a plausible explanation(s) for the impaired tissue and cell functions induced by the VSC. Fig. 2 demonstrates a remarkable effect produced by CH_3SH on the epithelial cell surface of fresh mucosal specimens mounted in the depicted perfusion apparatus (Fig. 1) and subjected to conditions described

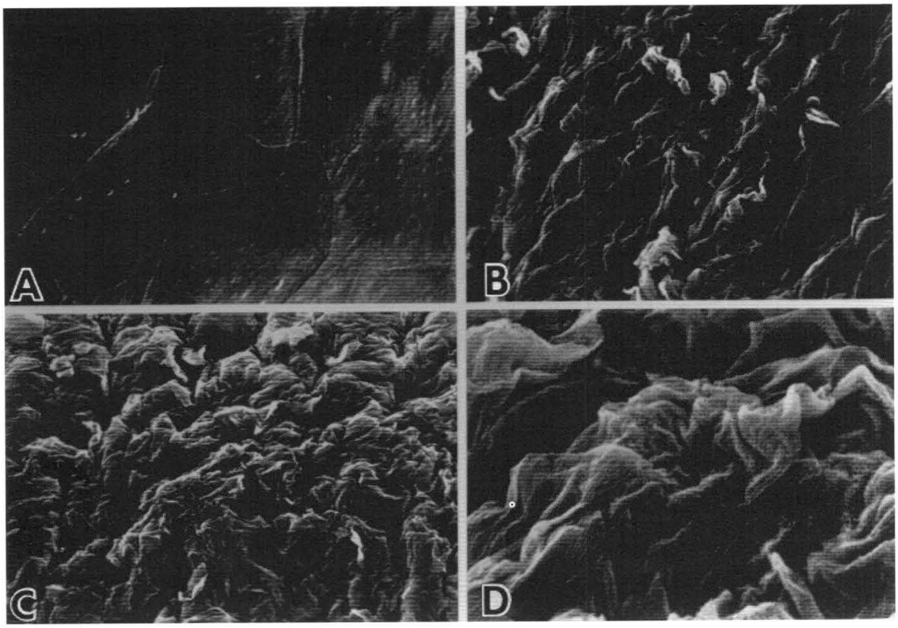


Fig. 2: Scanning electron microscopic examination of 1 mm thick mucosal specimens exposed for 0 to 8 h to 10 ng CH_3SH/ml of air/CO_2 atmosphere. A, depicts control tissue, while B, C and D represent tissues exposed to CH_3SH at 37°C for 2, 4 and 8 h, respectively.

for the permeability studies. Whereas the control tissues (A) displayed a relatively smooth epithelial cell surface, the SEM examination of 2 h exposed specimens revealed marked disruption and alteration of the epithelial tissue surface by CH_3SH. Some cells were exfoliated, and the visible surface appeared highly irregular with formation of deep intercellular spaces ascribed to a loss of intercellular matrix. These effects were further magnified during longer 4 and 8 h periods of incubation.

Parallel permeability and SEM investigations were also conducted with vesicle preparations derived from *Porphyromonas gingivalis* (P.g.). These vesicles are minute outer membrane fragments expelled by the organism into the culture medium. Treatment of the mucosal outer epithelial surface with 100 µg of the harvested vesicular preparation produced similar affects as CH_3SH on the permeability and surface appearance of the exposed tissues. A timed study (0 to 12 h) of FITC fluorescent immunolocalization of vesicles showed vivid evidence of penetration by vesicles of the mucosa to the basement membrane level. The penetration, as indicated by the intensity of

fluorescence, increased with time.

There is a sound rationale for determining the combined effect of vesicles and CH_3SH because P.g. is a prolific producer of CH_3SH and has been implicated in the etiology of periodontal disease, and as the vesicles exhibit proteolytic activity that requires a reducing agent such as dithiothreitol or CH_3SH for optimal activity. It was found that the combined effect of CH_3SH and vesicles was more pronounced than obtained with either agent alone (Fig. 5).

The TEM images of control (Fig. 3A) and test systems (Fig. 3B) in Fig. 3 show dramatic alterations in the basal membrane tissue layer of mucosa treated with vesicles/CH_3SH (Fig. 3B). Whereas the control tissues (Fig. 3A) showed a continuous intact basal membrane and well-defined lamina lucida and lamina densa, the test systems exhibited a virtually total disruption of the membrane. The lamina lucida totally disappeared and the remains of lamina densa was represented by disorganized dark material.

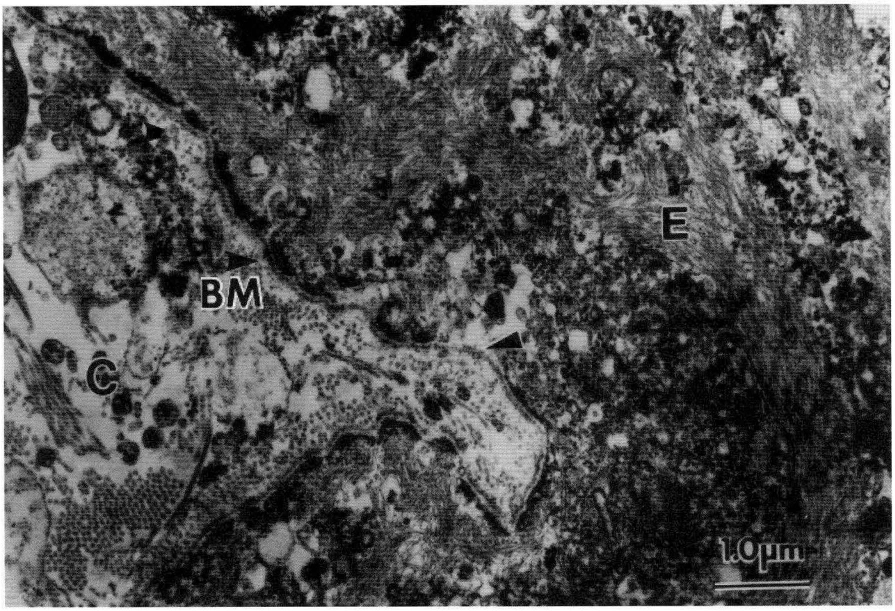


Fig. 3A: Transmission electron microscopic image of control tissue section that displays a well-defined and continuous basal membrane.

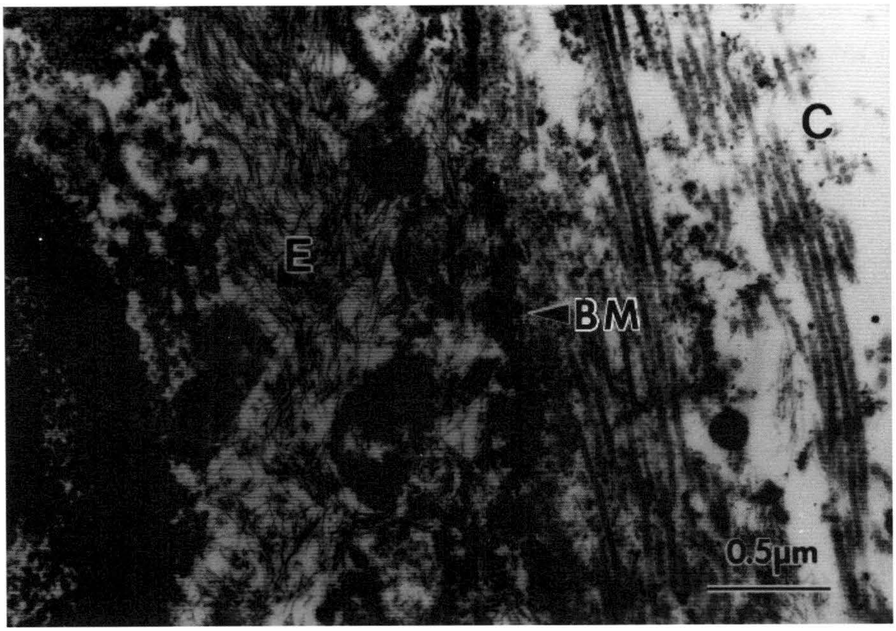
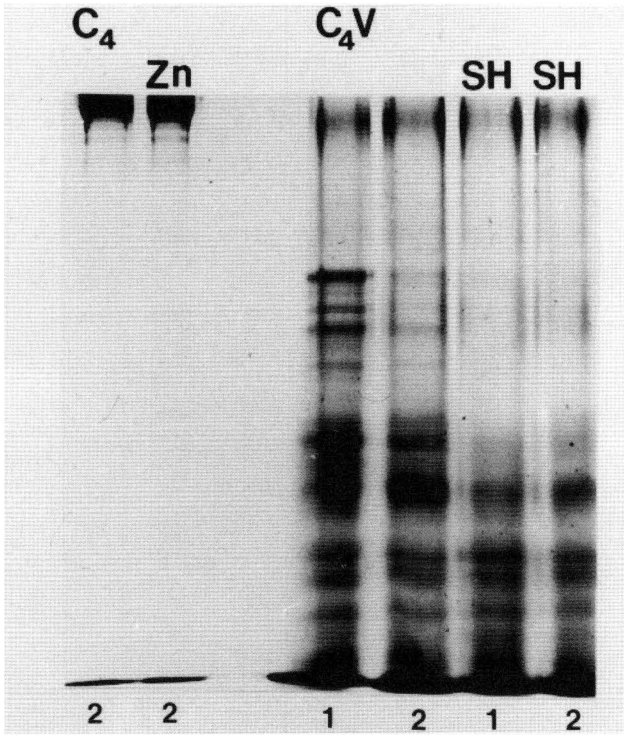


Fig. 3B: Transmission electron microscopic visualization of test tissue section after 4 h exposure to vesicles/CH_3SH.

The SDS/PAGE electrophoresis and immunofluorescence were employed to discern the effect of vesicles/CH_3SH on type IV collagen, fibronectin and laminin, the principal components of the basal membrane. The SDS/PAGE analyses of "in vitro" systems employing type IV collagen (Fig. 4), fibronectin and laminin as substrates yielded clear evidence that vesicles/CH_3SH rapidly and efficiently degraded all three substrates and that the Zn^{++} ion impeded the degradation process.

Immunochemical technique was also employed to elicit information as to whether the vesicles/CH_3SH combination possesses the potential to degrade type IV collagen, fibronectin and laminin of mucosal specimens. Using FITC fluorescent-labelled antibodies against these three components, it was deduced that the vesicles/CH_3SH treatment exerted a degradative effect on all tested substrates and thereby provided a plausible explanation for the observed decrease in the effectiveness of the basal membrane diffusion barrier. These results correlate with SDS/PAGE analysis and complement the previously cited permeability, SEM, and TEM observations.



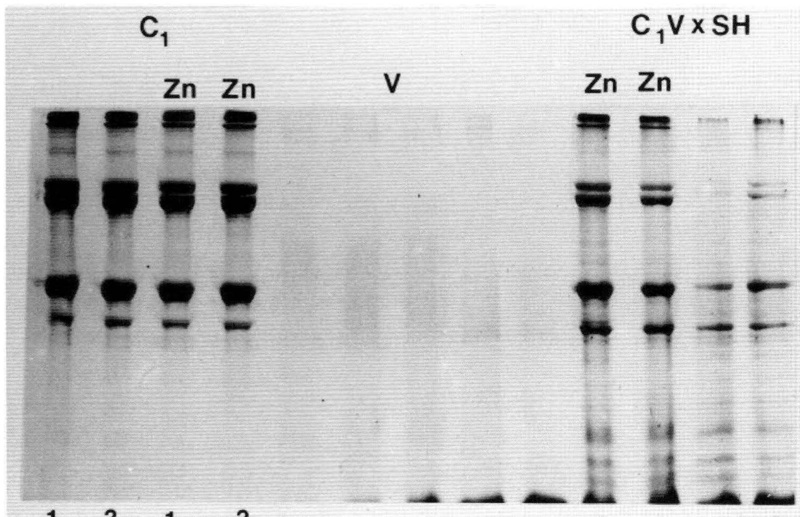
H*

L^+ 1 2 3 4 5 6 7 8

H* denotes hours of exposure (1 or 2 h).
L^+ denotes lane numbers (1 tot 8).

Fig. 4: The SDS/PAGE electrophoresis profiles of the effect of vesicles (V)/CH_3SH (SH) on type IV (C_4) collagen. Lanes 1 and 2 represent control systems in presence (lane 2) and absence (lane 1) of Zn^{++}. Lanes 3 and 4 illustrate that the vesicles did not contribute to any of the displayed protein bands, while lanes 5 to 8 demonstrate the effect of vesicles/CH_3SH after 1 (lanes 5 and 7) and 2 h (lanes 6 and 8) of reaction. It is evident that the combined effect is more pronounced than that produced by vesicles alone.

The TEM analysis displayed in Fig. 3B revealed a marked loss of intercellular matrix and a decrease and thinning of collagen fibers in the connective tissue layer of the treated mucosa. Johnson, in his chapter, disclosed that CH_3SH caused a reduction in both type I and III collagens in periodontal fibroblast cell cultures. The observed effect on type III collagen can be anticipated as the



H* 1 2 1 2 1 2 1 2

L+ 1 2 3 4 5 6 7 8 9 10 11 12 13

H* denotes hours of exposure (1 or 2 h).
L+ denotes lane numbers (1 to 13).

Fig. 5: The SDS/PAGE profiles of type I collagen (C_1) reacted for 1 and 2 h with vesicles/CH_3SH at 37°C. Lanes 3 and 4 are representative of control systems in presence and absence (lanes 1 and 2) of Zn^{++}. Lanes 5 to 9 indicate absence of protein bands ascribed to vesicles, while lanes 10 to 13 are indicative of degradation in presence (lanes 10 and 11) and absence (lanes 12 and 13) of Zn^{++} ion. The depicted figure infers that Zn^{++} has no effect on the peptide chains of type I collagen (lanes 3 and 4), that the vesicles did not account for any of the displayed protein bands, and that Zn^{++} inhibited the degradation of type I collagen in the test "in vitro" test systems.

three a1 chains of its molecule are held together by hydrogen-bonding and three disulphide linkages which are susceptible to cleavage by CH_3SH which would enhance solubilization of type III collagen fibers. As type I collagen comprises 80 to 85% of the collagen content of gingival tissue and periodontal ligament and virtually 100% of bone collagen, SDS/PAGE analysis of an "in vitro" study was conducted to ascertain the susceptibility of type I collagen to vesicles/CH_3SH. It is evident from the Fig. 5 that type I collagen is readily degraded by vesicles and that the process is enhanced by CH_3SH. For a more complete picture, the effect of the vesicles and CH_3SH on oral mucosa in the described studies should also be conducted on proteoglycans which are important components of all three tissue layers of mucosa. This phase

of the study has been delayed due to the unavailability of good proteoglycan samples.

To date, the cited experimental findings suggest the probable mechanism(s) leading to the destruction of periodontal tissues in periodontal inflammatory disease. The mechanisms were simulated in "in vitro" studies conducted on oral mucosa and cell cultures exposed to CH_3SH and/or P.g. vesicles. Most of the demonstrated biochemical processes leading to tissue destruction were inhibited by Zn^{++} ions.

Microbiology and Breath Odour

Oxygen Depletion by the Oral Microbiota and its Role in Oral Malodour Formation

*I. Kleinberg, D.P.M. Codipilly and D.Y. Globerman**

Introduction

Oxygen is available continuously to the various microbial communities that grow as bacterial biolayers on the oral hard and soft tissue surfaces throughout the human mouth. Some oxygen comes from inspired and expired air but most comes from saliva where the oxygen is in solution and hence more readily available to the oral bacteria for their metabolism. The oxygen level (PO_2) in freshly secreted whole saliva is about 65 mm Hg but the same saliva only 30-60 seconds later (just before swallowing) shows an oxygen level of about 35 mm of Hg (1). This can drop even lower, if plaque is allowed to accumulate on tooth and gingival surfaces by avoiding oral hygiene for several days. Such large and rapid decreases in oxygen levels can be attributed to the rapidity of the oxygen depletion that occurs when saliva and bacteria come in contact with each other (2) and the bacteria oxidatively degrade the carbohydrate and nitrogenous substrates normally present in the saliva (3, 4). Especially significant is the fact that the relatively small amount of resting saliva that passes through the mouth during each swallowing cycle, about 0.2 to 0.4 ml, (5) comes in contact with a comparatively large number of bacteria (about 2.5 mg wet weight of which about 2/3 is on the tongue) (6) spread over a relatively large surface area (about 214 cm^2) (5).

A number of microbial and biochemical changes have been demonstrated in experiments where all the plaque is removed from a tooth surface and then allowed to reform by avoiding oral hygiene. These changes include bacterial succession from a more aerobic/Gram-positive flora to one that is more anaerobic and Gram-negative (7, 8). Associated with this microbial shift is a drop in the plaque PO_2 (1), a decrease in the plaque oxidation-reduction (E_h) potential (9) and an increase in oral malodour and gingivitis-periodontitis development (10, 11).

Accordingly, the main focus of this paper is on various factors and processes that affect oxygen depletion and how such depletion affects and interrelates with E_h reduction and oral malodour generation by the Gram-negative anaerobes of the oral microbiota.

---

* I. Kleinberg, D.P.M. Codipilly and D.Y. Globerman, Department of Oral Biology and Pathology, State University of New York, Stony Brook, New York 11776-8702, USA.

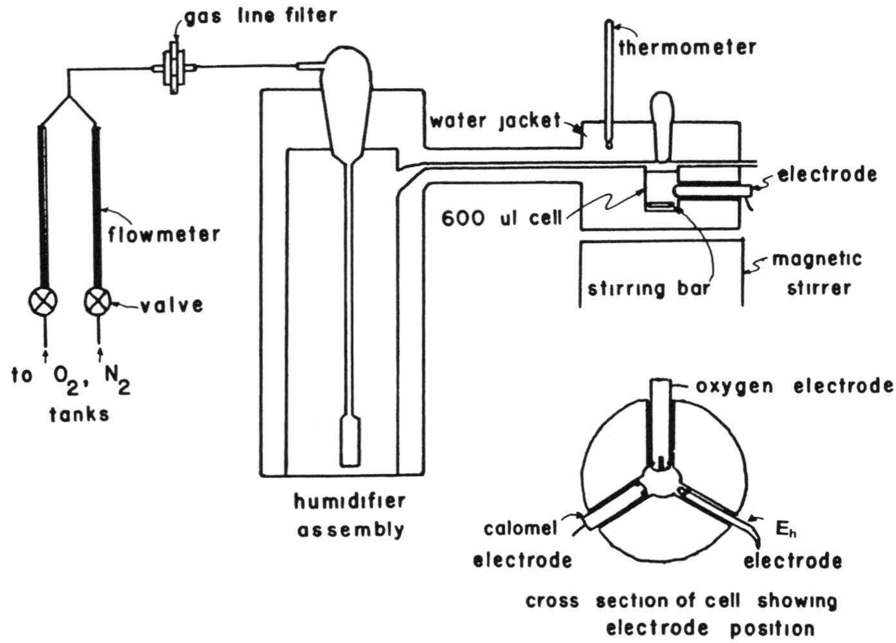


Fig. 1: Apparatus for controlling the ambient oxygen available to incubating salivary sediment mixtures during measurement of their PO_2 and E_h with oxygen and platinum electrodes. PO_2 uptake by the mixed bacteria in the sediment was stimulated by provision of glucose and/or salivary supernatant. Sediment was at 16.7 % (v/v), glucose at 0, 2.8 or 28 mM and supernatant at 0 or 33.3 % (v/v). Ambient oxygen levels were regulated by adjusting the proportions of the oxygen and nitrogen gaseous sources.

Determination of the oxygen depletion capacity of the oral bacteria

A series of experiments was carried out in our laboratory to assess the oxygen utilization capability of the oral bacteria with and without stimulation of oxygen consumption by exposure to glucose and/or salivary supernatant which was shown earlier to contain a variety of oxidizable substrates (3, 4, 12). An assessment of this capability was achieved with the apparatus shown in Fig. 1 in which oxygen levels could be adjusted to provide a wide range of ambient oxygen atmospheres at the same time that oxygen consumption by the oral mixed bacteria in salivary sediment (or plaque) incubation mixtures could be examined with oxygen and other electrodes. The oxygen levels tested were 0, 150, 360 and 760 mm Hg (the last two are much higher than the oral bacteria would normally encounter). Results are shown in Table 1. The first column in this table shows these ambient PO_2 levels and the last column

shows the corresponding rates of oxygen provided to the bacteria as a result. The other columns show the various rates of oxygen consumption observed, with the shaded area delineating those substrate conditions where oxygen consumption is necessarily limited to the level of ambient oxygen available because the incubating bacteria are using oxygen at less than their maximum rates. Under these conditions, a PO_2 of 0 mm Hg is achieved. Under all of the other substrate conditions shown, there is an excess of ambient oxygen and hence the PO_2 remains above zero. Note that only at an ambient PO_2 of 760 mm Hg, namely with pure oxygen flowing through the apparatus at atmospheric pressure, was a PO_2 of zero in all test cases prevented.

Another set of experiments with the same system and similar incubation mixtures examined the effects on the E_h of the incubations when glucose and the substrates in saliva are oxidized by the oral bacteria both in the absence

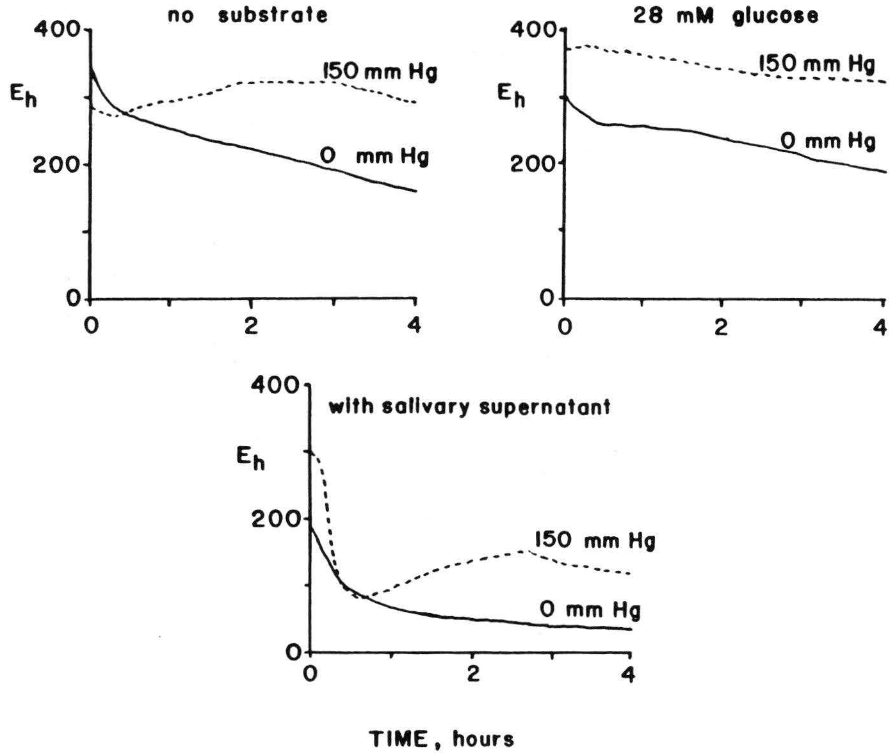


Fig. 2 : Oxidation-reduction (E_h) levels when salivary sediment mixtures were incubated with and without glucose or salivary supernatant, and with ambient oxygen at 0 or 150 mm Hg.

and in the presence of oxygen at 150 mm Hg. The E_h was measured with a platinum electrode in one of the ports of the diagram shown in Fig. 1. In all cases, oxygen presence favored a higher E_h, but most importantly, glucose favored either no change or a slight E_h elevation whereas saliva favored a substantial drop (Fig. 2). These observations are in agreement with an earlier report from our laboratory where it was also shown that (i) glucose degradation favors E_h elevation and (ii) salivary supernatant, together with the substrates it provides, favors an E_h decrease. What was significant in this study was the observation that there is no malodour formation with glucose but significant malodour with salivary supernatant (11). Earlier, Miller (13) and McNamara, Alexander and Lee (14) found that the acidic pH produced when glucose is fermented by the oral bacteria inhibits malodour formation. However, if in these experiments, the pH is buffered at 7.0, presence of glucose will still favor malodour inhibition. This can be attributed to the failure of the E_h in this and the above unbuffered experiments to decrease as well.

Table 1 : Maximum rates of oxygen uptake by salivary sediment at different ambient PO_2 levels.

Ambient PO_2 (mm Hg)	Oxygen uptake (µl O_2/mg/h)						Oxygen availability (µl O_2/mg/h)
	- supernatant			+ supernatant			
	glucose (mM)			*glucose (mM)*			
	0	2.8	28.0	0	2.8	28.0	
0	0*	0*	0*	0*	0*	0*	0
150	1.36	2.28*	2.28*	2.28*	2.28*	2.28*	2.28
380	2.42	4.57	5.09	5.00*	5.75*	5.75*	5.75
760	2.28	2.44	4.88	7.42	7.42	8.51	11.50

** Circled area includes the mixtures where oxygen uptake by the sediment bacteria is restricted by availability.*

In vivo O_2 and E_h changes during plaque re-formation experiments

As pointed out in the introduction, levels of both PO_2 and E_h decrease as plaque accumulates in plaque re-formation experiments. Explanation for the

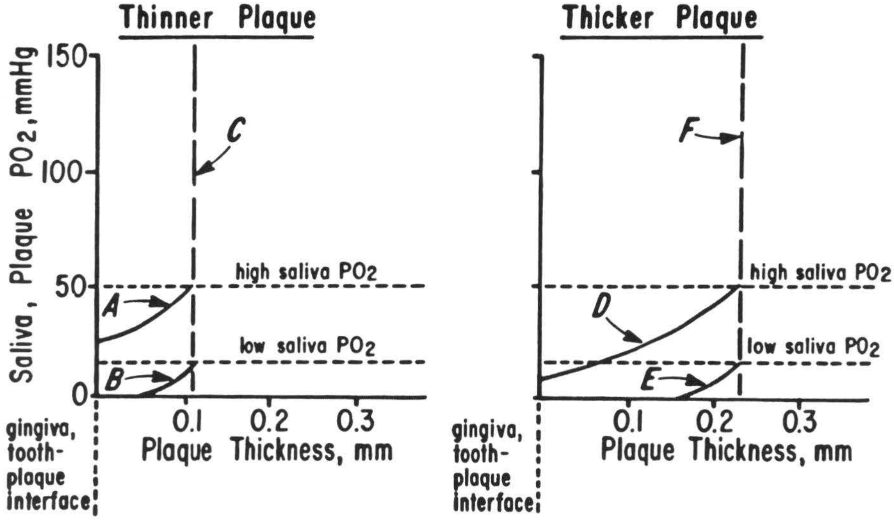


Fig. 3 : Effect of plaque thickness and salivary oxygen levels on the PO_2 within the plaque at different distances from the tooth or gingival mucosa surface. In a thin plaque, only a narrow inner region of the plaque is anaerobic at low salivary oxygen levels, whereas at high salivary oxygen levels, no part of the plaque is without oxygen. In contrast, in a thicker plaque, a greater proportion of the plaque is likely to be anaerobic, especially at low salivary oxygen levels. C and F are lines indicating examples of plaque thickness in the two diagrams; where plaque oxygen lines A and B meet C, and plaque oxygen lines D and E meet F, salivary oxygen and plaque surface oxygen levels would theoretically be the same. However, because of the extremely rapid rate of oxygen use by the plaque bacteria, plaque surface and plaque interior oxygen levels are likely to be lower than shown (16).

lowering of the PO_2 was provided by Stralfors (15) who also explained how the plaque PO_2, oxygen consumption rate of the plaque bacteria and plaque thickness were interrelated (Fig. 3). An important point that emerged was that achievement of a PO_2 of zero mm Hg is very dependent on the relative magnitudes of these parameters. For example, if the ambient oxygen level of a plaque was 150 mm Hg, i.e. atmospheric, a plaque would have to be thicker than about 0.3 mm before its inner regions could be anaerobic, whereas at the level of saliva just before swallowing, it would only have to be 0.1 mm thick. Interproximal and embrasure plaque is often thicker than these examples so that one can expect the plaque in these less accessible sites to be anaerobic most of the time and thus favor the conditions and bacteria leading to a low E_h Gram-negative anaerobic bacteria, malodour and gingivitis-periodontitis.

A related point that emerges from the work of Stralfors, is that above a certain thickness, plaque should be considered as a two (but not necessarily uniform) zoned structure (16, 10). One zone would be devoid of oxygen, truly anaerobic and by and large, always have a PO_2 of zero, thus favoring the most anaerobic bacteria. The other zone would experience oxygen presence, with the surface the most aerobic and locations distant from the plaque surface progressively less so (Fig. 3). It would be reasonable to expect fluctuation between oxygen presence and absence in this second zone because of ambient oxygen variation Facultative bacteria would best thrive in such an aerobic/anaerobic environment and their predominance in the oral microbiota would attest to that. To some degree, the odour generating capabilities of these two oxygen conditions can be modelled and compared by examining sediment (or plaque) incubation mixtures without and with an oil layer covering. This will exclude environmental oxygen and the results for such an examination are presented two sections below.

Progressive decrease in the E_h was seen in plaque reformation studies by Kenney and Ash (9) using platinum electrodes. Such a decrease was also observed by Katayama, Susuki and Okada (17) using methylene blue and triphenyltetrazolium chloride oxidation-reduction indicator dyes. These dyes have E_0 values of about +11 MV and -80 MV, respectively. The Kenney and Ash investigation (9) not only showed E_h reduction; it also showed emergence of a more anaerobic Gram-negative biota and that a lower E_h is related to periodontitis development. Evidently, progressive bacterial succession, PO_2 depletion, E_h lowering, emergence of a Gram-negative biota, oral putrefaction and oral malodour go hand-in-hand. Particularly important is the possibility that oxygen depletion can make it easier for a greater plaque E_h lowering to occur and favour growth of harmful, odour-producing Gram-negative anaerobes.

It has been known for many years that decreases in E_h may involve more than one oxidation-reduction system to accept the electron flow associated with bacterial growth and metabolism of energy producing oxidizable substrates (18). Since oxygen is an excellent electron acceptor, it could be the first oxidant of a two-stage system (Fig. 4). In such circumstances, oxygen depletion would serve as a means of removing the first stage of the two-step E_h lowering system; in a sense, this removal of an oxygen barrier to substantial E_h reduction, would then become like an oxygen free anaerobic chamber or apparatus used to grow anaerobic bacteria. An electron acceptor with a lower E_0 than oxygen, such as various sulphur compounds could provide the second stage reduction directly and hence the lower E_h needed by the stricter anaerobes than might otherwise be possible (18). This could

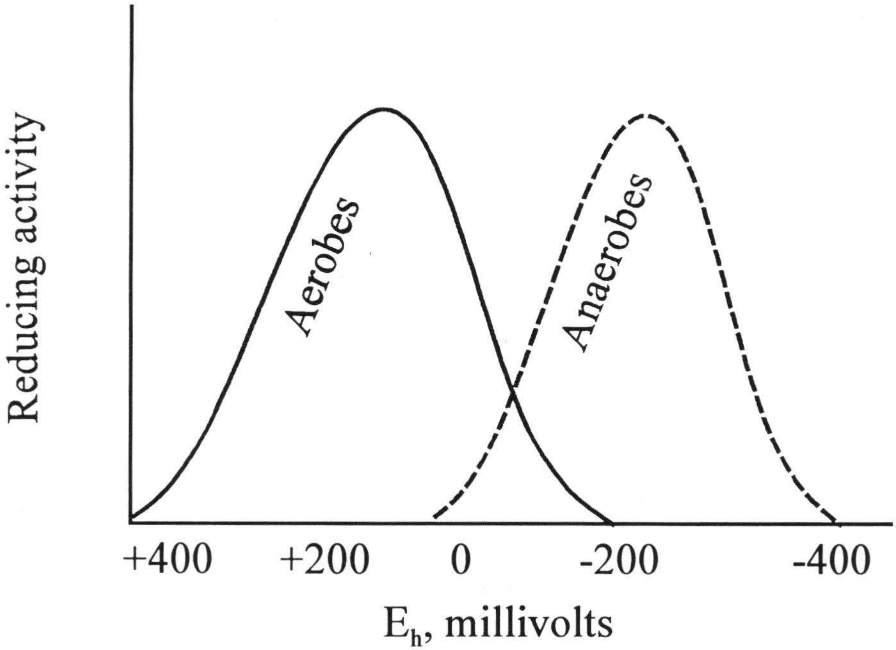


Fig. 4 : Hypothetical reducing activities of aerobic and anaerobic bacteria in a two-stage reduction of the E_h (18).

be one of the reasons that anaerobes begin to emerge at about the same time that oxygen is depleted from the plaque and more anaerobic bacteria are then seen as the E_h continues to fall (9). Hewitt (18) has proposed that two-stage E_h reduction is necessary when a large E_h decrease is needed to provide conditions for the more anaerobic bacteria to grow and metabolize. In such a case, rapid oxygen depletion would be necessary to provide a low E_h in an environment where a continual supply of oxygen is present, as is the case within the oral cavity.

Biochemical processes involved in oxygen depletion

For oxygen consumption by the oral bacteria to occur, there also has to be substrate utilized. Several earlier studies have indicated that the oxidizable substrates involved in the oral cavity fall into two main categories (i) carbohydrate derived from the diet and from saliva, the latter mostly coming from the sugar moieties of its glycoproteins (3, 2) and (ii) several of the common animo acids, mainly glutamate, proline, ornithine and perhaps some

arginine (4). The level of free amino acids in saliva is generally low; hence, most of the amino acids oxidized come from salivary peptides and proteins upon their degradation by the oral bacteria. These four amino acids are probably linked through pathways leading to glutamate (19). It is the most dominant amino acid in the intracellular pool of free amino acids (20) and one that is central to a number of cellular intermediary metabolism processes (19).

Oxidation of carbohydrate and amino acids results in significant oxygen depletion. With carbohydrate, hydrogen peroxide is formed by aerobic glycolysis along with acetic acid by certain of the oral bacteria (21). It is this process that evidently favors the E_h elevation seen with glucose. The bacteria mainly responsible for peroxide formation are the most prominent streptococci found in the mouth, viz. *Streptococcus sanguis, S. oralis* and *S. mitior* (21). However, there are also numerous and extremely rapid peroxide users present in the plaque biota. These include *Hemophilus parainfluenzae* and *H. segnis, Neisseria sicca, Actinomyces viscosus* and *Staphylococcus aureus* (21), which are rich in the enzyme, catalase. As a result, it is easy for their collective consumption capability to overwhelm the collective ability of the peroxide formers to produce peroxide. Consequently, peroxide presence is difficult to achieve (22) and any bactericidal effect of accumulated peroxide is largely lost. In essence, because different bacteria contribute to the two peroxide processes, their relative numbers would determine whether there will be any hydrogen peroxide in excess.

Decrease in the E_h by the bacteria in salivary sediment mixtures is favored when salivary supernatant is counteracted by the presence of fermentable sugars (11). In the case of the latter, this is only so under aerobic conditions when it is possible for the bacteria to produce peroxide along with acetate from pyruvate under aerobic conditions.

In a recent study, Traudt and Kleinberg (12) surveyed a large number of the numerically prominent oral bacteria for their individual abilities to use oxygen during their catabolism of a wide variety of sugar, amino acid and other substrates. The data showed a large number of the Gram-positives and only certain of the Gram-negatives could oxidize sugars. Remarkable was the fact that the oxygen consumption by Gram-positives was restricted to sugar substrates : they were unable to use amino acids oxidatively (12). Presumably, this reflected their aerobic glycolysis capabilities (21). In contrast, Gram-negatives were the exclusive oxygen utilizers with amino acids. These bacteria included *Hemophilus, Neisseria* and *Veillonella* species. Basically, they made it possible for Gram-negative anaerobes such as the Fusobacteria and Bacteroides which are the primary bacteria involved in oral malodour, to grow and metabolize. Interestingly, the bacteria that

proved to be the most active oxygen depleters were those involved in early plaque colonization. These included mainly the *Streptococci* and *Actinomyces* of the Gram-positive members of the oral microbiota and *Hemophilus* and *Neisseria* of the Gram-negatives. Earlier, the main amino acid substrates involved in oral malodour were identified as cysteine, cystine, methionine, tryptophan and ornithine (11).

Role of ambient aerobic and anaerobic conditions on malodour production

When a layer of mineral oil was used to exclude atmospheric air in the classical sediment type incubation experiment (11) and compared to a similar incubation open to the atmosphere, significant malodour was generated by the bacteria under both conditions. For these experiments, salivary sediment was prepared from stimulated whole saliva in 10 x 75 mm test-tubes as described earlier (11) and incubated with salivary supernatant with and without a covering layer of mineral oil. Two series of mixtures were prepared. One consisted of salivary sediment at 16.7% (v/v), supernatant at 33.3% (v/v) and a total volume of 750 µl. The other was exactly the same but with a mineral oil covering of 750 µl. The mixtures were then incubated for 24 hours at 37°C. At the beginning and at regular intervals throughout the incubations, a mixture was removed from each series and the head space and the incubation solutions were analyzed for malodour organoleptically on a 0 to 4 scale, and for volatile sulphur compounds (VSC) with the Halimeter (Interscan Corporation, Chatsworth, CA) (23). This instrument measures volatile sulphur compounds, mainly hydrogen sulphide. Also analyzed was indole/skatole production using Kovac's Indole reagent (Difco, Detroit) along with decrease in the E_h employing a platinum electrode.

The results are shown in Figs. 5 to 8. As expected, head space Halimeter VSC and organoleptic odour assessments (Figs. 5 and 6) were essentially absent in the oil layered incubation as compared with in the one without oil. However, when incubation mixtures were transferred with pipettes to new test-tubes at each time interval, both oil and no oil covered mixtures showed substantial increases in both VSC and organoleptic head space values (Figs. 5 and 6).

This clearly showed that the malodour volatiles were in an over-saturated state and with little disturbance would be lost from solution. When a glass rod was inserted into these transferred incubations and then smelled by a single examiner, the malodour increased progressively and almost equally with time in both (Fig. 7). Interestingly, Halimeter VSC values rose faster

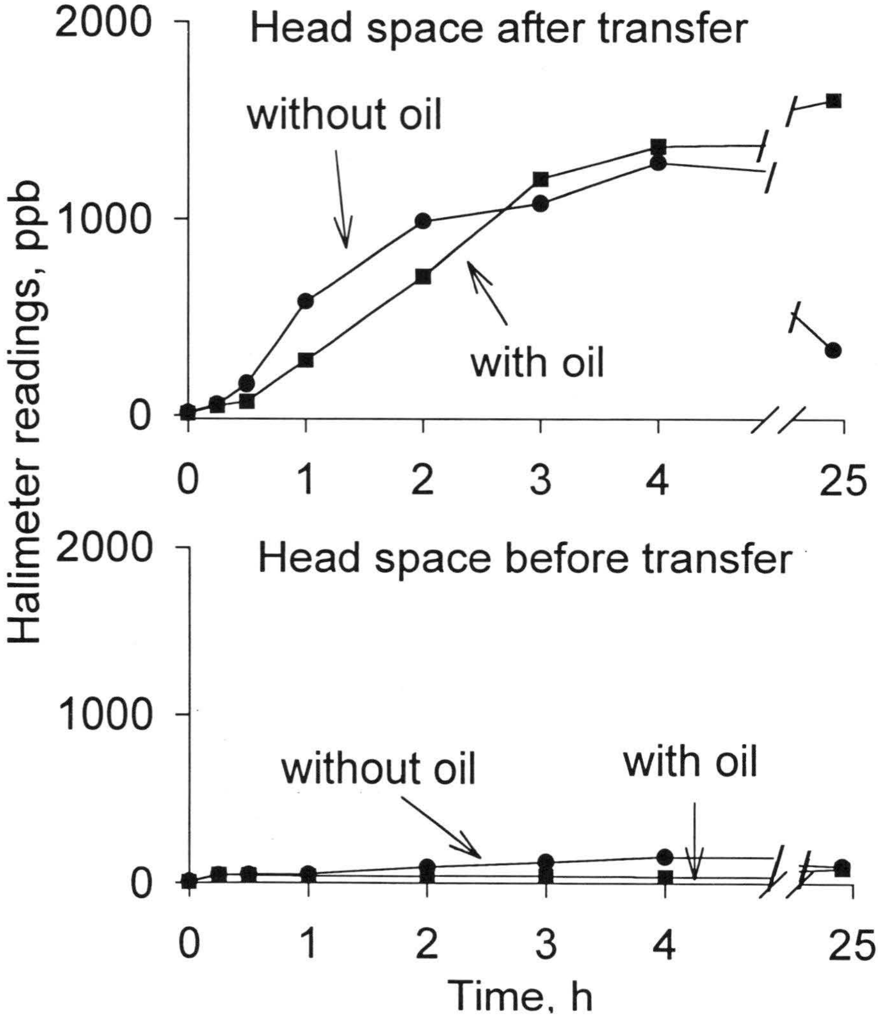


Fig. 5 : Effect of oxygen availability on Halimeter assessment of volatile sulphur compound (VSC) production in incubated salivary sediment mixtures containing sediment bacteria at 16.7 % (v/v) and salivary supernatant at 33.3 % (v/v). Head space VSC was determined in the incubation mixtures before and after their transfer with a pipette (minus the mineral oil) to fresh 10 x 75 mm test-tubes.

initially in the absence of oil, reached a peak at 4 hours and then fell significantly thereafter. In contrast, when oil was present, the Halimeter VSC values continued to rise and showed no decrease, thus reflecting the trapping of the volatile sulphur compounds generated under the oil. In contrast, the indole/skatole in the incubation mixtures progressively increased but was

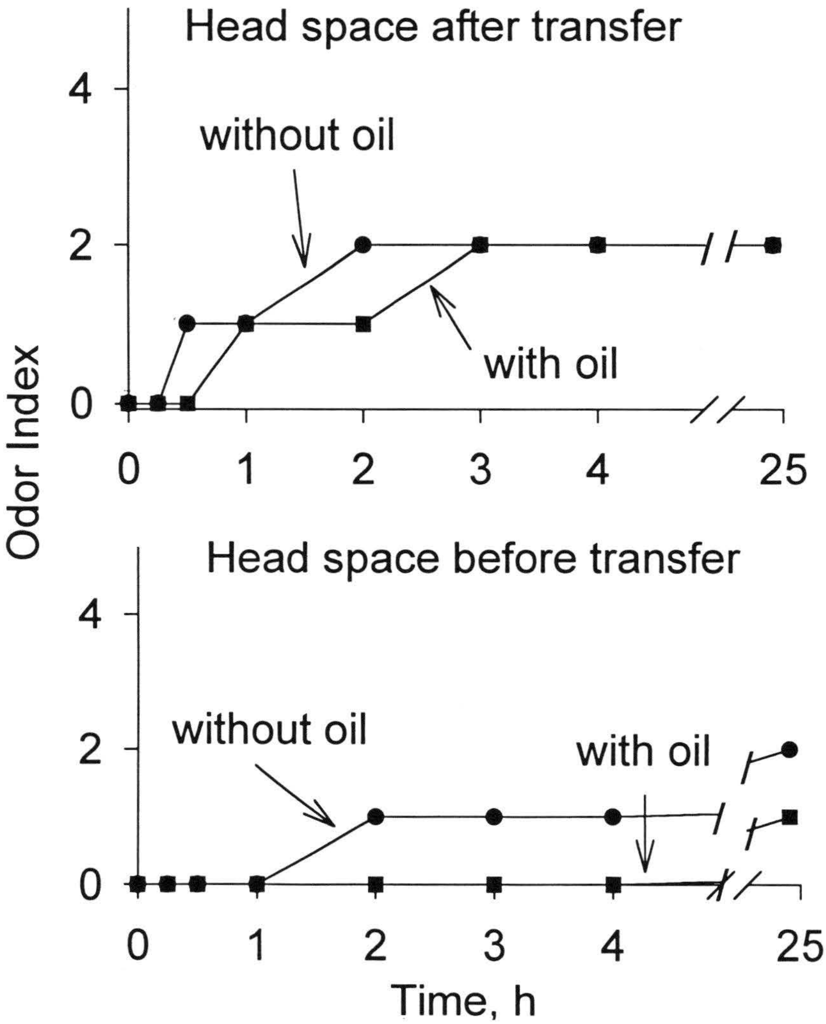


Fig. 6 : Effect of oxygen availability on the organoleptic assessment of odour production in incubated salivary sediment mixtures containing sediment bacteria at 16.7 % (v/v) and salivary supernatant at 33.3 % (v/v). As in Fig. 5, odour assessment was done before and after incubation mixture transfer to eliminate presence of the mineral oil.

considerably greater in the absence of the mineral oil (Fig. 8). This suggests that oxygen favors indole/skatole formation and their contribution to the malodour. The E_h decreased equally in both oil and non-oil covered incubations.

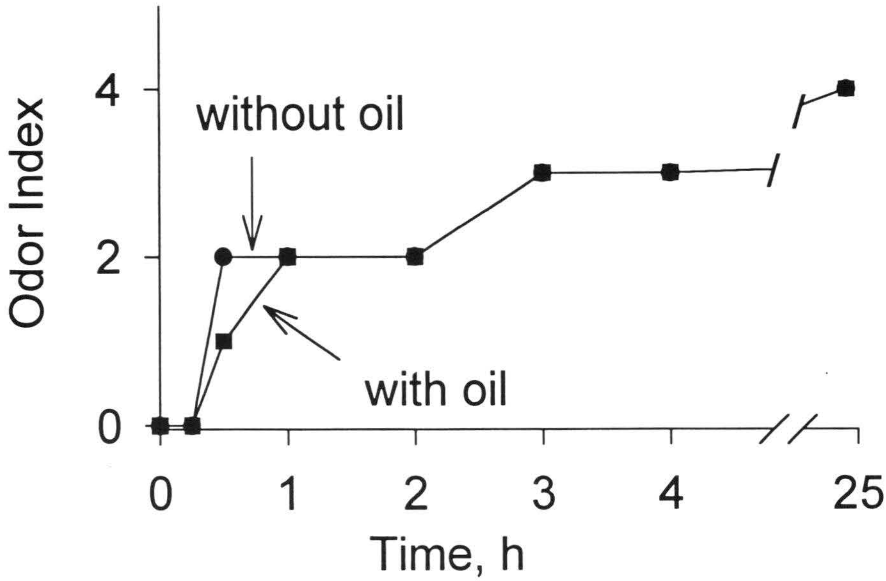


Fig. 7 : Effect of oxygen availability on organoleptic assessment (rod method) of odour production in incubated salivary sediment mixtures containing sediment bacteria at 16.7 % (v/v) and salivary supernatant at 33.3 % (v/v). A glass rod was inserted into the transferred incubation mixtures in Fig. 6 and then smelled and assessed on a 0 to 4 scale.

From these results, it would appear that VSC odour is favored more by the presence of anaerobic conditions, whereas indole/skatole odour is favored by more aerobic conditions. This leads to an interesting speculation. Namely, in a two-zone plaque, one might find both VSC and indole/skatole odour occurring with the anaerobic portion favoring the former and the oxygen depleted portion favoring the latter. What is rather interesting, is that disturbance of the incubation supernatant easily releases malodorous products, which evidently as pointed out above, must be present in an over-saturated state. This would support our earlier view that loss of over-saturated odoriferous volatiles can readily take place if there is oral mucosal drying, particularly of the hard palate, as can occur especially in individuals during speech or other such conditions (11). This evaporative phenomenon might be an important factor favoring bad breath.

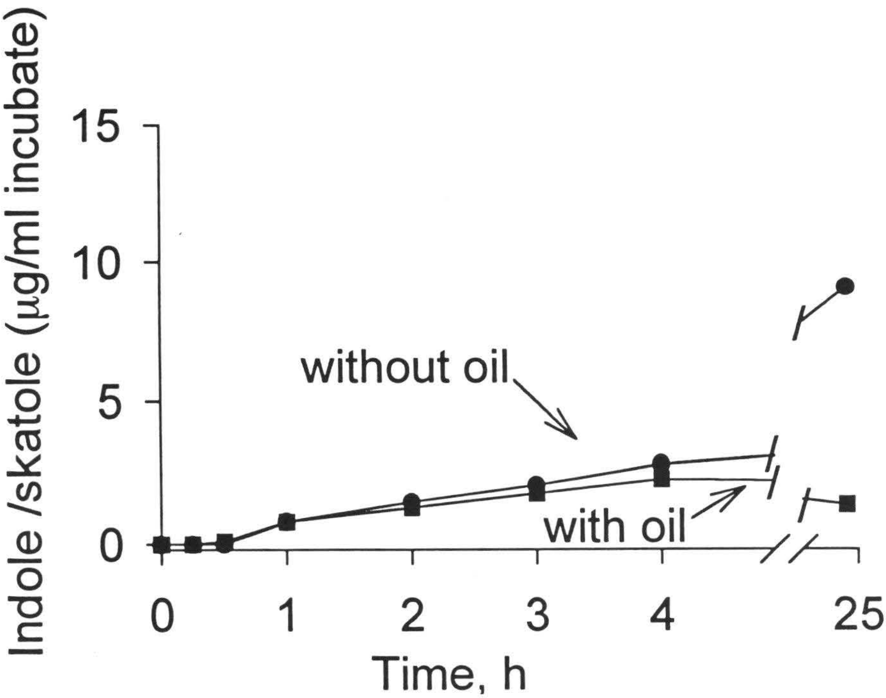


Fig. 8 : Effect of oxygen availability on indole/skatole production in incubated salivary sediment mixtures containing sediment bacteria at 16.7 % (v/v) and salivary supernatant at 33.3 % (v/v).

Concluding remarks

Collectively, the results in this report and those of Stralfors (15) and Hewitt (18), suggest that oxygen depletion may play a complex but important role in malodour production. Depletion is probably a significant factor in determining the extent to which the E_h falls during amino acid degradation, which in turn, is an important determinant of the types of bacteria that are able to grow and whether oral putrefaction and malodour generation will occur. Oxygen depletion and hence oxygen availability may control the types of odoriferous compounds produced and therefore could play a part in the nature of the malodour. In this regard, the type of malodour would be very different, if VSC production were favored over the formation of indole/skatole or vice versa.

Acknowledgements

The secretarial assistance of Ms. Patricia Calia and diagram preparations by Dr. H.W. Kaufman are most gratefully acknowledged.

References

1. Globerman D.Y. and Kleinberg I. (1979) Intra-oral PO_2 and its relation to bacterial accumulation on the oral tissues. In: Kleinberg I., Ellison S.A. and Mandel I.D. (Eds) *Saliva and Dental Caries.* Information Retrieval, New York and Washington, pp. 275-291..
2. Korayem M.R., Traudt M. and Kleinberg I. (1991). Oxygen uptake and its relation to pH in a human salivary system during fermentation of glucose. *Archs oral Biol* 35:759-764.
3. Molan P. and Hartles R.L. (1971) The nature of the intrinsic salivary substrate used by the human oral flora. *Archs oral Biol* 16:1449-1462.
4. Korayem M.R., Westbay G. and Kleinberg I. (1990) Constituents of salivary supernatant responsible for stimulation of oxygen uptake activity of the bacteria in human salivary sediment. *Archs oral Biol* 35:145-152.
5. Collins L.M.C. and Dawes C. (1987) The surface area of the adult human mouth and thickness of the salivary film covering the teeth and oral mucosa. *J Dent Res* 66:1300-1302.
6. Loesche W.J. and Gibbons R.J. (1966) Influence of nutrition on the ecology and cariogenicity of the oral microflora. In:Nizel A.E. (Ed) *The Science of Nutrition and its Application in Clinical Dentistry.* Saunders, Philadelphia and London, 2nd ed, pp. 305-317.
7. Ritz H.L. (1967) Microbial population shifts in developing human dental plaque. *Archs oral Biol* 12:1561-1570.
8. Löe H., Theilade E. and Borglum-Jensen S.B. (1965) Experimental gingivitis in man. *J Periodont Res* 36:177-187.
9. Kenney E.B. and Ash M.M. (1969). Oxidation-reduction potential of developing plaque, periodontal pockets and gingival sulci. *J Periodontol* 40:630-633.
10. Kleinberg I. and Westbay G. (1992) Salivary and metabolic factors involved in oral malodor formation. *J Periodontol* 63:768-775.
11. Kleinberg I. and Codipilly, D.P.M. (1995) The biological basis of oral malodor formation. In: Rosenberg M. (Ed) *Bad Breath : Research Perspectives.* Ramot Publishing-Tel-Aviv University, Israel, pp 13-39.
12. Traudt M. and Kleinberg I. (1996). Stoichiometry of oxygen consumption

and sugar, organic acid and amino acid utilization in salivary sediment and pure cultures of oral bacteria. *Archs oral Biol* (In press).

13. Miller W.D. (1890) The Micro-organisms of the Human Mouth. S.S. White, Philadelphia (reprinted in 1973 by Karger, Basel).
14. McNamara T.F., Alexander J.F. and Lee M. (1972) The role of microorganisms in the production of oral malodor. *Oral Surg Oral Med Oral Pathol* 34:41-48.
15. Stralfors A (1956) An investigation of the respiratory activities of oral bacteria. *Acta Odontol Scand* 14:153-186.
16. Kleinberg I. and Westbay G. (1990) Oral malodor. *Crit Rev Oral Biol Med* 1:247-259.
17. Katayama T., Suzuki T. and Okada S. (1975) Clinical observation of dental plaque maturation. Application of oxidation-reduction indicator dyes. *J Periodont Res* 46:610-613.
18. Hewitt L.F. (1950) Oxidation-Reduction Potentials in Bacteriology and Biochemistry, Livingstone, Edinburgh, pp 96-136.
19. Stryger L. (1988) Biochemistry. 3rd edn, W.H. Freeman, New York, pp 503.
20. Singer D.L. and Kleinberg I. (1983). The free amino acids in human dental plaque. *Archs oral Biol* 28:873-878.
21. Ryan C.S. and Kleinberg I. (1995). Bacteria in human mouths involved in the production and utilization of hydrogen peroxide. *Archs oral Biol* 40:753-763.
22. Kraus F.W., Nickerson J.F., Perry W.I. and Walker A.P. (1957) Peroxide and peroxidogenic bacteria in human saliva. *J Bacteriol* 73:727-735.
23. Rosenberg M., Septon I., Eli I., Bar-Ness A., Gelernter I., Brenner S. and Gabbay J. (1991) Halitosis measurement by an industrial sulfide monitor. *J Periodontol* 62:487-489.

The Tongue Microbiota and Tongue Surface Characteristics Contribute to Oral Malodour

*E. H. De Boever and W. J. Loesche**

Introduction

Proteolytic degradation of proteins, peptides and mucines by the oral microbiota adherent to the tongue and to a lesser extent the teeth, contribute to oral malodour (1-4). Over 80 species resident in the subgingival plaque can, at least *in vitro*, be considered malodorous because they produce volatile sulphur compounds (4, 5), fatty acids or both. Some of the most prominent odour forming bacterial species, including members of the *Treponema, Prevotella, Porphyromonas* and *Selenomonas* genera, have been associated with periodontitis, suggesting a link between the latter and halitosis. In addition, oral sites other than the periodontal pocket and gingival tissues are also involved in odour-production. One such site is the dorsal surface of the tongue, where large amounts of bacteria are present in fissures and between mucosal papillae. These fissures and crypts may create an environment where the microorganisms are well-protected from the flushing action of the saliva and where oxygen levels are low, which in turn promotes the development of an anaerobic microbiota at these sites. This paper reviews findings from our laboratory and other investigators in support of the primary role of the tongue microbiota in the etiology of oral malodour.

The periodontitis-halitosis relationship

The association between periodontitis and oral malodour has received wide attention. There is ample evidence to show that volatile sulphur compounds (VSC) levels in the mouth correlate with the extent of periodontal breakdown (6,7), and that the amounts of sulphur volatiles in the breath increase with the number and depth of the periodontal pockets (8,9). Furthermore, bleeding pockets also contain higher levels of VSC than shallow, healthy sites (10). By inserting lead strips into periodontal pockets (11), or by using a sensitive gas-chromatographic technique which allows VSC-measurements at

* E.H. De Boever and W.J. Loesche, Oral Health Sciences, School of Dentistry, University of Michigan, Ann Arbor, Michigan 48109-1078, USA.

individual periodontal sites (8,10), it can be demonstrated that the periodontal pocket is a place of VSC-production in these patients.
Thus, it is conceivable that periodontal breakdown is a causative factor in odour formation, at least in people with periodontitis.

Tongue coating and oral malodour

There are, however, a number of periodontally healthy people who suffer from chronic halitosis and whose bad breath cannot be explained by the relationship described above. Bosy *et al.* examined 127 patients, the majority of which presented to the clinic with subjective complaints of oral malodour (11). Several variables were measured in these patients, including VSC-levels, organoleptic mouth and tongue odour ratings, and estimates of gingival inflammation and periodontitis. When the subjects were divided into four groups according to halitosis levels and periodontal health, there was no significant association between the presence of halitosis and periodontitis, and the VSC-concentrations and organoleptic odour scores were very similar among the four groups (Table 1). Findings from this and other studies (7) demonstrate that a substantial proportion of people with complaints of oral malodour do not have periodontal infections.

Table 1 : Evaluation of the halitosis-periodontitis relationship : evidence of a periodontally healthy subpopulation with oral malodour.

	Periodontitis[a] Halitosis[b]	No Periodontitis Halitosis	Periodontitis No Halitosis	No Periodontitis No Halitosis
Number of subjects (%)	23 (18)	52 (41)	14 (11)	38 (30)
VSC Peak (ppb)	135.7	110.4	73.9	69.1
OR-Mouth[c]	3.5	3.4	1.3	1.7
OR-Tongue[d]	2.7	2.7	1.7	1.7

Association of Halitosis with Periodontitis χ^2=0.208 P-value=0.65

a: Periodontitis = 1 or more pockets of ≥ 5 mm
b: Halitosis = organoleptic exceeding 3 on an ordinal scale
c: OR-Mouth = Full mouth organoleptic odor measurement
d: OR-Tongue = Tongue odor organoleptic measurement

Adapted from Bosy et al. Periodontol 1994; 65:37-46

In periodontally healthy individuals with chronic malodour, a combination of conventional oral hygiene and tongue brushing is twice as effective as toothbrushing alone (7), indicating that the dorsal surface of the tongue is a likely place for proteolytic activity in these patients. Indeed, a growing body

of research indicates that tongue coating also plays a significant role in odour formation in periodontally diseased patients (11-16). Tongue coating is composed of blood components and other nutrients, large amounts of desquamated epithelial cells and bacteria, suggesting that it has the putrefactive capacity to produce large amounts of volatile sulphur compounds and other malodorous molecules. Yaegaki measured the amount of the tongue coating from patients with and without periodontitis, and determined the amounts and types of VSC produced by this coating (12,13). While subjects with periodontitis had significantly higher levels of VSC in their breath, they had on average 6 times more coating on their tongues, and this tongue coating was responsible for 60% of the total VSC production in these patients. The majority of the VSC was identified as methylmercaptan, which is organoleptically more offensive than H_2S or dimethyl sulphide (17). Bosy *et al.* (11) explored the relationship of VSC with tongue odour in their patient population and concluded that the dorsal surface of the tongue is an important site of odour production in the mouth.

Studies from our own laboratory confirm these findings. We examined 54 individuals with and without complaints of oral malodour as part of a study to investigate the association between self-reported halitosis, certain tongue surface features, (specifically the presence of tongue coating and or fissures), and selected bacterial groups colonizing the tongue. In order to optimize organoleptic and quantitative measurements, the subjects were instructed to refrain from eating, drinking, smoking, chewing gum and rinsing their mouths for at least two hours before each appointment, and not to brush, floss or use commercial mouth rinse on the day of the examination. In addition to clinical parameters, such as organoleptic odour ratings, VSC level measurements, bleeding scores and periodontal status, we evaluated the presence of tongue coating and fissures. We will summarize the results of this study some of which has been previously published (15).

We were unable to establish a relationship between the levels of sulphur compounds in the breath and gingival health. Neither the extent nor the severity of gingival bleeding was associated with VSC-production, suggesting that the periodontal tissues were not the primary source of VSC-formation in our study population (Table 2). In contrast, tongue odour showed a positive linear relationship with the levels of VSC in the breath ($r=0.561$; $p < .05$), and with full mouth organoleptic odour ($r=0.662$; $p < .005$) (15). Whole mouth odour, tongue odour and VSC levels were markedly higher in individuals complaining of bad breath who also had a visible tongue coating, fissures or objectionable odour coming from their tongue (i.e. organoleptic score 2) (Table 3). A complaint of oral malodour significantly increased the subject's

probability of having clinical malodour (as defined by an organoleptic rating exceeding 2), and of having tongue coating or fissures. High mouth odour scores were associated with the presence of coating or deep fissures on the tongue (Table 4). Not only are these findings in agreement with recent reports citing the tongue as a primary site of oral odour production, they also indicate that certain tongue surface features might promote the colonization of the tongue with odour producing bacteria.

Table 2 : Relationship between VSC-levels in the breath and gingival health.

	Tertile of VSC (ppb)			p-value
	Lower (0-138)	Middle (138-240)	Upper (>240)	
Subjects (n)	18	18	18	
VSC (ppb)	107.3 ± 22.9	178.3 ± 24.6	436.0 ± 181.1	< 0.001[c]
OR	1.6 ± 0.6	2.4 ± 0.6	2.9 ± 1.0	< 0.001[c]
Complaints (n)	3/18	8/18	9/18	n.s.[d]
Coating (n)	4/18	11/18	11/18	< 0.03[d]
Fissures (n)	1/18	6/18	8/18	< 0.03[d]
Bleeding Index[a]	40.8	42.8	39.3	n.s.[c]
Bleeding Severity[b]	22.2	19.4	13.5	n.s.[c]

a: Percentage of sites bleeding upon probing or with PBS ≥ 2
b: Percentage of sites bleeding with flow of blood along the gingival margin
c: Kruskall-Wallis test
d: Chi-Square analysis

Table 3 : Coating, fissures and odour in subjects with malodour complaints.

	Subjects (n)	OR Mouth	OR Tongue	VSC (ppb)
Coating Yes	15	3.1	3.3	349.4
No	5	2.2*	1.4*f*	224.1
Fissures Yes	8	3.4	3.4	403.1
No	8	2.0*	2.4*f*	215.7
Tongue Odor Yes	15	3.1*	3.4*f*	378.7*
No	5	2.0	1.0	135.9

OR Mouth = full mouth organoleptic score;
OR Tongue = Organoleptic rating tongue
* P-value ≤ 0.05 (Mann-Whitney U-test)
f P-value < 0.01 (Mann-Whitney U-test)

Table 4 : Association between odours, complaints of halitosis, and tongue parameters (n=54).

Explored Association	χ^2	df	P-value
Complaints and Mouth Odour[a]	8.145	1	0.004
OR[b]	12.90	3	0.005
Coating	6.075	1	0.014
Fissures	7.819	1	0.005
Mouth Odour and Coating	11.63	1	0.001
Fissures	7.831	1	0.005
Organoleptic Rating and Coating	19.15	3	0.001
Fissures	11.87	3	0.008

a: mouth odour = organoleptic rating > 2
b: OR = organoleptic rating

Role of tongue bacteria in halitosis development

Despite the compelling evidence that the tongue coating is probably responsible for the majority of the VSC found in the mouth air, a number of questions remain unanswered. It is not known, for instance, which bacterial species in the tongue coating are involved in this odour production. There is some evidence to suggest that the tongue biota contains some species which are similar to the odour producing periodontal plaque flora (18, 19). These studies, however, looked at a small number of individuals without complaints of halitosis. No one has simultaneously monitored the tongue biota and malodour levels in individuals with complaints of oral malodour and correlated the specific bacterial species with malodour.

To address this issue, we included a component in the study outlined above that would allow us to investigate the association between self-reported halitosis and selected bacterial groups colonizing the tongue. In addition to information on various halitosis and clinical parameters, we collected tongue coating material from each patient for bacteriological evaluation. To account for varying amounts of coating on each subject's tongue, and to make comparisons across groups possible, we transferred a standardized inoculum of tongue coating material to transport media, and subsequently plated ten-fold serial dilutions onto various media. Given the vast number of bacterial species present in the mouth and the fact that most species or groups of bacteria colonizing the tongue have not yet been characterized, it would be a time consuming task to easily identify or characterize the tongue microbiota using classical culturing techniques. We circumvented this

problem by dividing the oral microbiota into segments corresponding to known physiological states (e.g. anaerobic biota, facultative organisms, saccharolytic biota), and mimicked these states by carefully choosing the culture media and growth conditions. In addition, we tested the tongue coating material for the presence of *Treponema denticola, Porphyromonas gingivalis* and *Bacteroides forsythus*, three odour producing bacterial species, which are unique in that they are capable of hydrolyzing the synthetic trypsin-like substrate benzoyl-DL-arginine-2-naphthylamide (BANA). By means of the BANA™ test (20), we could monitor these and other BANA-positive and possibly malodorous species in the subgingival plaque and in samples taken from the tongue coating.

Table 5 : Relationship between oral malodour variables and bacterial colonization of the tongue

	Subjects (n)	BANA(+) Tongue Sample (n)	Bacterial Density (CFU/sample)	Facultative Bacteria[d] (%)
Complaints Yes	20	16	13.2×10^6	63.5
No	34	23	0.5×10^6	117.4
P-value		< 0.08[b]	< 0.002[a]	< 0.01[a]
Malodour Yes[c]	17	17	13.0×10^6	70.2
No	37	26	1.6×10^6	110.0[c]
P-value		< 0.02[b]	< 0.002[a]	< 0.04[a]

a: Mann Whitney U-test

b: Chi-Square Association

c: Malodour = organoleptic odour rating > 2

d: Percentage Facultative Bacteria = $\frac{\text{CFU on Metronidazole supplemented Media}}{\text{CFU on Non Selective Media}} \times 100$

Our results indicate that the presence of tongue coating was related to the bacterial density or total number of bacteria in the tongue coating sample. Given that we used a standardized inoculation volume for each subject (i.e., one wire loop of coating material), differences in total counts retrieved from the sample probably reflect differences in the number of bacteria actually colonizing the subjects' tongues. Participants who had coated tongues harbored over 25 times more bacteria per unit volume than subjects who did not have coated tongues. Similarly, subjects with high organoleptic odour scores (organoleptic rating 3 or 4) were found to have ten-fold higher bacterial counts than their non-malodorous counterparts, suggesting that bacterial

load on the tongues of individuals with malodour may be ten times greater than in subjects without appreciable halitosis (Table 5). These increased counts per unit volume (density) do not take into account the fact that the individuals with malodour had more coating material than those without malodour, so that the overall bacterial load on the tongue is considerably higher than the 10-25 fold increase in densities noted.

We explored the relationship between VSC-concentrations and bacteriological variables by dividing the study group into tertiles of quantitative odour measurements (Table 6). A positive linear trend was observed between VSC-levels in the breath and the number of subjects with BANA-hydrolyzing bacteria on their tongue, while no difference could be seen between the three groups in terms of the number of BANA-positive interproximal plaque samples. The importance of BANA-activity on the tongue in odour formation was further illustrated by the fact that BANA-hydrolyzing bacteria on the tongue, but not in interproximal plaque samples, was associated with complaints of malodour and high odour scores (Table 5).

Table 6 : Relationship between VSC-concentrations and bacterial variables.

	Tertile of VSC (ppb)			
	Lower (0-138)	Middle (138-240)	Upper (> 240)	p-value[b]
Subjects (n)	18	18	18	
VSC (ppb)	107.3 ± 22.9	178.3 ± 24.6	436.0 ± 181.1	<0.001
BANA(+) Sites	4.6/6[c]	4.3/6	4.8/6	
BANA(+) Tongue Sample	12/18[d]	14/18	17/18	
Bacterial Density[a]	0.9×10^6	8.6×10^6	6.2×10^6	<0.02
Anaerobic/Aerobic Flora	1.5	1.7	2.1	

a: number of colony forming units (CFU) per sample

b: Kruskall-Wallis test

c: number of positive sites/number of tested sites

d: number of subjects with positive tongue sample/total number of subjects in the group

The proportion of the biota recovered from the tongue coating that was anaerobic was highest in the group with the highest VSC-concentrations in their breath, which was consistent with our hypothesis that the anaerobic microbiota are responsible for the odour formation (Table 6). Similarly, the

percentage of the tongue microbiota that was facultative, was significantly lower in subjects with a complaint of halitosis, and in individuals with mouth odour scores of 3 or 4 (Table 5). Note that facultative counts, obtained from a medium supplemented with 5 μg/ml metronidazole, were compared to the growth on standard media to calculate the percentage of the flora that was facultative. In some cases, the numbers of organisms retrieved from the standard medium were lower than those counted on the metronidazole-supplemented agar, explaining why some of the values exceed 100%.

These findings complement a previously published study in which we used a 0.12% chlorhexidine gluconate mouthrinse to demonstrate that reducing the oral microbiota on the tongue and other mucosal surfaces caused reductions in volatile sulphur measurements and organoleptic odour ratings (15) (Table 7). After treatment, the average organoleptic odour score was less than 1, which is considered non-objectionable, and significant reductions in the bacterial density and in the number of BANA-positive tongue samples were observed. The reduction in the percentage of tongue coating samples exhibiting BANA-activity was consistent with Bosy's findings that chlorhexidine treatment reduced tongue BANA scores (11).

Table 7 : Halitosis and bacteriological parameters at baseline and after treatment.

	Baseline	After Treatment	Percent Change	P-value[b]
VSC[a] (ppb)	309.4 ± 241	82.2 ± 32.0	-73.3	< 0.002
OR-Oral[a]	2.9 ± 0.9	0.9 ± 0.6	-68.6	< 0.001
OR-Tongue[a]	2.7 ± 0.9	0.6 ± 0.8	-77.8	< 0.001
Total Counts (CFU)	9.4 x 10^6	2.4 x 10^6	-74.0	< 0.03
% Facultative Organisms	69.1	111.4	+61.2	
% BANA(+) Tongue Samples	68.8	18.8	-50.0	< 0.003
% BANA(+) Plaque Samples	90.6	41.7	-48.9	< 0.003

a: mean ± SEM
b: Paired t-test analysis

Adapted from De Boever et al. JADA 1995; 126:1384-1393

These observations suggest that the BANA™ test can help identify some of the bacterial species that are responsible for, or that biochemically resemble

the organisms that produce odour on the tongue. We also saw a shift towards more facultative, and conceivably less odorous, microbiota (Table 7), but we were unable to correlate odour measurements or chlorhexidine treatment with the asaccharolytic portion of the tongue microbiota. Taken together, these data lend support to the idea that the Gram-negative anaerobic microbiota in the oral cavity and in particular on the tongue, are associated with oral malodour production.

Conclusion

As Rosenberg (21) pointed out, treatment strategies for halitosis should be aimed at reducing the levels of odour-producing organisms on the tongue and in the dental plaque. The research presented in this paper indicates that in the majority of the patients suffering from bad breath, the bacterial load on the tongue is indeed a major reservoir for the malodorous microbiota, and in these cases treatment should target the odour-producing tongue microbiota. Elimination or reduction of these microbiota could be achieved in a number of ways, including mechanical debridement of the tongue with a stick or toothbrush, and/or by means of antimicrobial mouth rinses specifically aimed at controlling the flora associated with oral malodour production.

References

1. Tonzetich J. (1978) Oral malodor: an indicator of health status and oral cleanliness. *Int Dent J* 28:309-319.
2. Kleinberg I. & Westbay G. (1990) Oral Malodor. *Crit Rev Oral Biol Med* 1:247-259
3. McNamara T.F., Alexander J.F. & Lee M. (1972) The role of microorganisms in the production of oral malodor. *Oral Surg* 34:41-48.
4. Persson S., Claesson R. & Carlsson J. (1989) The capacity of subgingival species to produce volatile sulfur compounds in human serum. *Oral Microbiol Immunol* 4:169-172.
5. Persson S., Edlund M.B., Claesson R. & Carlsson J. (1990) The formation of hydrogen sulfide and methyl-mercaptan by oral bacteria. *Oral Microbiol Immunol* 5:195-201.
6. Sato H., Ohkushi T., Tsunoda T. & Sato T. (1980) A study of the mechanism of halitosis occurrence in periodontal patients. *Bull Tokyo Dent Coll* 21:271-278

7. Kaizu T., Tsunga M., Aoki H. & Kimura K. (1978) Analysis of volatile sulphur compounds in mouth air by gas chromatography. *Bull Tokyo Dent Coll* 19:43-52.
8. Persson S. (1992) Hydrogen sulfide and methyl mercaptan in periodontal pockets. *Oral Microbiol Immunol* 7:378-379.
9. Yaegaki K. & Sanada K. (1992) Volatile sulfur compounds in mouth air from clinically healthy subjects and patients with periodontal disease. *J Periodont Res* 27:233-238.
10. Coil J. & Tonzetich J. (1992) Characterization of volatile sulfur compounds production at individual gingival crevicular sites in humans. *J Clin Dent* 3:97-103.
11. Bosy A., Kulkarni G.V., Rosenberg M. & McCulloch C.A.G. (1994) Relationship of oral malodor to periodontitis: evidence of independence in discrete subpopulations. *J Periodontol* 65:37-46.
12. Yaegaki K. & Sanada K. (1992) Volatile sulfur compounds in mouth air from clinically healthy subjects and patients with periodontal disease. *J Periodont Res* 27:233-238.
13. Yaegaki K. & Sanada K. (1992) Biochemical and clinical factors enhancing oral malodor in periodontal patients. *J Periodontol* 63:783-789.
14. Yaegaki K. (1995) Oral malodor and periodontal disease. In: Rosenberg M. (Ed). *Bad Breath: Research Perspectives.* Ramot Publishing, Tel Aviv, Israel, pp 88-108.
15. De Boever E.H. & Loesche W.J. (1995) Assessing the contribution of anaerobic microflora of the tongue to oral malodor. *J Am Dent Ass* 126:1384-1393.
16. Miyazaki H., Sakao S., Katoh Y. & Takehara T. (1995) Oral Malodor in the general population of Japan. In: Rosenberg M. (Ed) *Bad Breath: Research Perspectives.* Ramot Publishing, Tel Aviv, Israel, pp 119-136.
17. Tonzetich J. & Ng S.K. (1976) Reduction of malodor by oral cleansing procedures. *Oral Surg Oral Med Oral Pathol.* 42:172-181.
18. Gordon D.F. & Gibbons R.J. (1966) Studies of the predominant cultivable microorganisms from the human tongue. *Archs oral Biol* 11:627-632.
19. Van Winkelhoff A.J., Van der Velden U., Winkel E.C. & De Graaff J. (1986) Black-pigmented bacteroides and motile organisms on oral mucosal surfaces in individuals with and without periodontal breakdown. *J Periodont Res* 21:434-439.
20. Loesche W.J., Bretz W.A., Kerschensteiner D., Stoll J.K., Socransky S.S., Hujoel P. and Lopatin D.E. (1990) The development of a diagnostic test for anaerobic periodontal infections based upon plaque

hydrolysis of benzoyl-DL-arginine naphtylamide (BANA). *J Clin Microbiol* 28:1551-1559.

21. Rosenberg M. (1990) Bad breath: diagnosis and treatment. *U Toronto Dent J* 3:7-11.

Assessment of Impressed Toothbrush as a Method of Sampling Tongue Microbiota

M. G. Hartley, M.A. El-Maaytah, C. McKenzie and J. Greenman[*]

The dorsum of the tongue can be divided into two symmetrical areas either side of the mid-line. The surface is papillated containing many pits and fissures which harbour a great diversity of oral microbes. One traditional method of sampling this uneven surface is to use a sterile cotton wool swab. An alternative approach is to use a sterile toothbrush impressed against the dorsal surface. These methods were compared by sampling the dorsum of the tongue (swab, left hand side (LHS); toothbrush, right hand side (RHS)) and processing the samples identically. Samples were diluted to 10^{-3} and plated onto fastidious anaerobe agar with 7% blood (incubated both anaerobically and aerobically) and the same media supplemented with vancomycin (2.5mg 1^{-1}) incubated anaerobically. An initial experiment was conducted to demonstrate the bilateral symmetry of the distribution of the microbiota using the toothbrush method. The results comparing the main groups of microbiota showed no significant differences between the LHS and RHS of the tongue. Aerobes, anaerobes and Gram- negative anaerobes had mean counts in log cfu cm^{-2} of 6.795, 7.60 and 7.12 respectively. Results comparing the swab and toothbrush sampling methods showed that their was no significant differences between the techniques. However, the toothbrush method was easier to perform, more reliably sampled a given area and could be used to sample the tongue more towards the posterior third. Moreover, less saliva was adsorbed to the brush compared to the swab and therefore it is likely that a higher proportion of local microbiota was isolated. The toothbrush method has now been used successfully to examine and identify the tongue microbiota.

Introduction

Tongue microbiota is attracting an increasing amount of interest as being a possible cause of unexplained persistent oral malodour. Several recent studies have examined its role in odour generation by experimenting with

* M.G. Hartley, M.A. El-Maaytah, C. McKenzie and J. Greenman, Bristol Oral Microbiology Unit, University of the West of England, Bristol, United Kingdom.

tongue scrapings (1, 2, 3). Whilst this has successfully illustrated the microbiota's ability to generate substantial odour, it gives little information on the component members of the microbiota. Other studies concerning the tongue microbiota have been published, but these have focused on specific aspects (4, 5); consequently little is known about normal tongue bacteria.

One of the biggest problems with analysing the tongue microbiota is sample taking. Traditionally a swab has been used for taking such samples. However as the tongue is highly convoluted, of unknown surface area and with relatively limited access, this may not be the best approach. An alternative method has been devised using a toothbrush as a sampling tool. This has several advantages over the more conventional swab; in particular the size of the toothbrush head is fixed. Therefore as long as the toothbrush is firmly pressed against the tongue without touching or dragging along other surfaces, and provided the pressure is constant between samples, then so is the area. As the area is fixed, the brush can be used with confidence to access areas that cannot be demarked with a clamp or other such device required to delineate the swab's contact area. The nylon bristles do not absorb saliva in the way a swab does, so that the sample is relatively uncontaminated by salivary bacteria. Moreover the nylon bristles may also penetrate the larger of the crypts towards the back of the tongue, where theoretically, rnicroenvironments exist which are more anaerobic and therefore harbour a microbiota with a greater odour generating potential.

The aim of this study was to assess the use of an impressed toothbrush as a method of sampling the dorsal surface of the tongue to isolate the microbiota. This method was compared to one using a swab. The method was then applied to investigate the distribution of recoverable microbiota from different areas of the tongue, to examine the variation of this microbiota over time for given individuals and also to record total recoverable numbers per unit area for a large group of subjects.

Methods

Subjects

The volunteers used for these experiments were recruited for a malodour trial and were therefore chosen for having consistently high or low oral malodour. They were all resident in Bristol LTK, in good general health and not taking antibiotics. Their oral health was assessed as good by a dental clinician, who

also ranked their level of malodour and took the samples. As much as possible they were allowed to continue their normal oral hygiene regime with the exception of the use of antimicrobial mouthrinses.

Sampling

For this study, samples taken from the front of the tongue were taken approximately 3 cm from the tip; samples defined as from the back of the tongue were taken as far back on the tongue as could be tolerated by the volunteer, approximately 7 cm from the tip. The toothbrush was applied firmly with gentle oscillatory pressure to encourage bristle penetration of the crypts, but without any lateral movement.

Sample processing

Samples were taken as early as possible within the working day, but at least 1 hour after oral activities such as eating and drinking. Samples were processed as quickly as possible, diluted in 1/4 strength Ringers (Oxoid) and plated onto fastidious anaerobe agar (FAA, lab M, Bury LTK), supplemented with 5% (v/v) horse blood. Duplicate sets of plates were incubated aerobically (3 days) and anaerobically (7 days) at 37°C to give counts of total recoverable aerobic and anaerobic microbiota respectively. The same media with the addition of vancomycin ($2.5 mgl^{-1}$, Sigma, Poole UK) were incubated anaerobically (7 days) to give an estimation of the number of Gram-negative anaerobes ($GNAnO_2$), which previous studies have suggested contain a high proportion of odour generating species. All counts were expressed as total counts; no further differentiation or identification was performed.

Results

The easiest way to compare the two sampling techniques was deemed to be by taking paired samples from comparable areas of each volunteers tongue, for example from left and right hand sides of the bilateral line, equidistant from the tip. Therefore the first experiment was designed to demonstrate that the tongue, akin to other body surfaces displays bilateral symmetry, with respect to the distribution of the microbiota.

Table I shows the bacterial counts of tongue microbiota from the right and

left-hand sides of the mid-dorsal line for 5 paired samples and 3 different total counts (anaerobic, aerobic and $GNAnO_2$), obtained using the toothbrush method. The counts were quite high, with a maximum in the order of 1 x 10^8 per cm^2 . There were no statistical differences between the samples for any of the counts (using a paired student's t-test), indicating bilateral symmetry of the tongue biofilm and consistency of the technique. Visual inspection of the recovery plates between each pair of samples failed to detect any differences with regard to the diversity of colony morphotypes observed.

Table 1: Bilateral symmetry of the tongue dorsum biofilm, viable counts obtained from paired toothbrush samples from the front of the tongue (n=5)

	RHS	LHS
anaerobes	7.77 ± 0.15	7.22 ± 0.22
	(7.24-8.08)	(6.86-8.10)
aerobes	6.94 ± 0.15	6.65 ± 0.25
	(6.48-7.32)	(5.86-7.28)
aerobes:anaerobes	1:7	1:4
Gram-negative	7.32 ± 0.13	6.92 ± 0.28
anaerobes	(6.84-7.60)	(6.30-7.64)
% of anaerobes	35.5	50.1

counts are log_{10}, cm^{-2} and given as mean ±se (range)

Table 2 : Comparison of bacterial counts obtained from comparable tongue samples (front using different sampling methods (n=5)

	Brush	Swab
anaerobes	7.75 ± 0.17	7.68 ± 0.21
	(7.16-8.18)	(7.14-8.31)
aerobes	7.10 ± 0.22	7.00 ± 0.16
	(6.86-8.10)	(6.53-7.55)
aerobes:anaerobes	1:4.5	1:5
Gram-negative	7.13 ± 0.17	7.22 ± 0.19
anaerobes	(6.65-7.51)	(6.72-7.66)
% of anaerobes	24.2	11.0

counts are log_{10} cm^{-2} and given as mean ±se (range)

Table 2 shows the bacterial counts obtained from either side of the mid-dorsal line, for 5 paired samples and 3 different counts, using the different sampling

techniques. The swab samples were taken 'free hand', so the area sampled was estimated. There were no statistical differences between the counts (by paired student's t-test) nor did the ratios appear to change. No observable differences were detected concerning the colonial morphotypes recovered from each individual using the different methods, suggesting that the same microbiota was being sampled regardless of technique.

Table 3: Bacterial counts from anterior and posterior areas of the tongue (n=10)

	Front	Back
anaerobes	7.37 ± 0.34	8.17 ± 0.28
	(5.74-8.71)	(6.65-9.79)
aerobes	6.71 ± 0.29	7.42 ± 0.21
	(5.26-7.88)	(6.20-8.53)
aerobes:anaerobes	1:5	1:6
Gram-negative	6.72 ± 0.41	7.59 ± 0.24
anaerobes	(6.65-7.51)	(6.12-8.62)
% of anaerobes	22.4	26.3

counts are log_{10} cm^{-2} and given as mean ±se (range)

The impressed brush method of sampling was used to explore the differences in the microbial flora between the front (approx. 3 cm from the tip) and further back (approx. 7 cm from the tip) on the tongue. The samples were taken along the mid-dorsal line. Table 3 shows the bacterial counts recovered from these sites for 10 paired samples, and 3 different counts. In quantitative terms the microbial levels were statistically different ($p < 0.05$), the microbiota at the back being almost 10 times greater than that at the front; however, this difference was equally apparent for all 3 types of count suggesting that in qualitative terms the microbial populations were very similar. Whereas visual comparison of colony morphology between the sites revealed greater variation than seen between sites from the previous experiments, there did not appear to be any constant pattern to these differences.

Sequential sampling of the same area on an individuals tongue was performed to ascertain the proportion of the total recoverable bacteria that was removed with each sample. Ten sequential samples were taken, each sample was taken with a fractionally smaller brush to reduce the chances of straying from the previously sampled area. Fig. 1 shows the regression of the anaerobic counts for 5 individuals who were examined in this manner. Apparently, even after 10 consecutive samples of the same site there was still a significant number of bacteria at that site. Some of this may have been

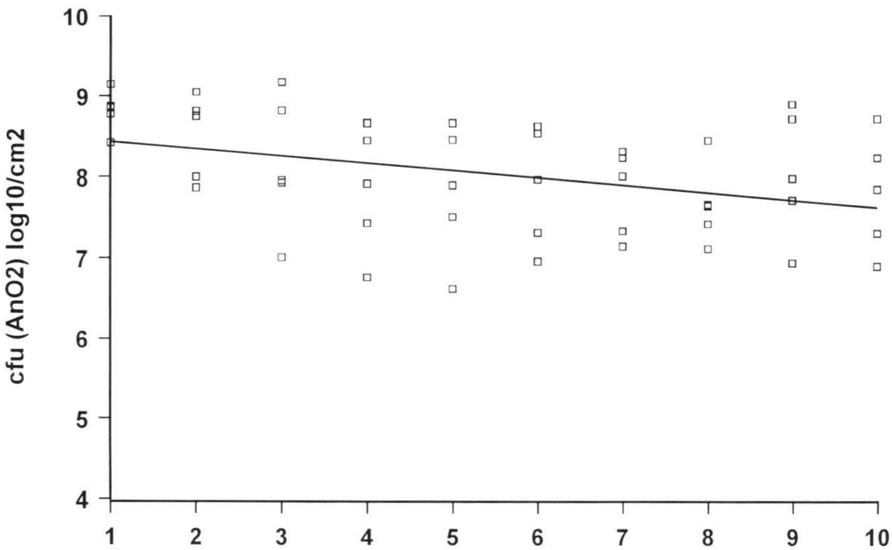


Fig. 1 : Anaerobic bacterial counts from repeated sampling (n=5)

the result of salivary contamination although the tongue was surrounded by absorbent gauze in an effort to limit this. This data suggests that as little as 1/10th of the resident microbiota is removed with each sample. Fig. 2 shows the change in the aerobic counts superimposed on Fig. 1. There was no trend towards a more anaerobic microbiota with continued sampling.

The impressed toothbrush method of sampling was used to obtain bacterial counts from a large number of subjects. Table 4 shows the counts for 153 samples taken from 48 individuals 'first thing', from sites towards the back of the tongue. For some of these samples, the aerobic count was omitted and replaced with microaerophilic counts, made from chocolatised blood and incubated for 3 days in an elevated CO_2 atmosphere (n=48). For a further 18 samples only the anaerobic and Gram-negative anaerobic counts were made. The counts recorded were high, up to 8.5 X $10^9 cm^{-2}$ for the anaerobes, and the range of the counts was large, over 3 orders of magnitude. Every sample contained both aerobes and anaerobes, usually within an order of magnitude of each other, but there was extensive variation in the proportion of Gram-negative anaerobes (see table 4).

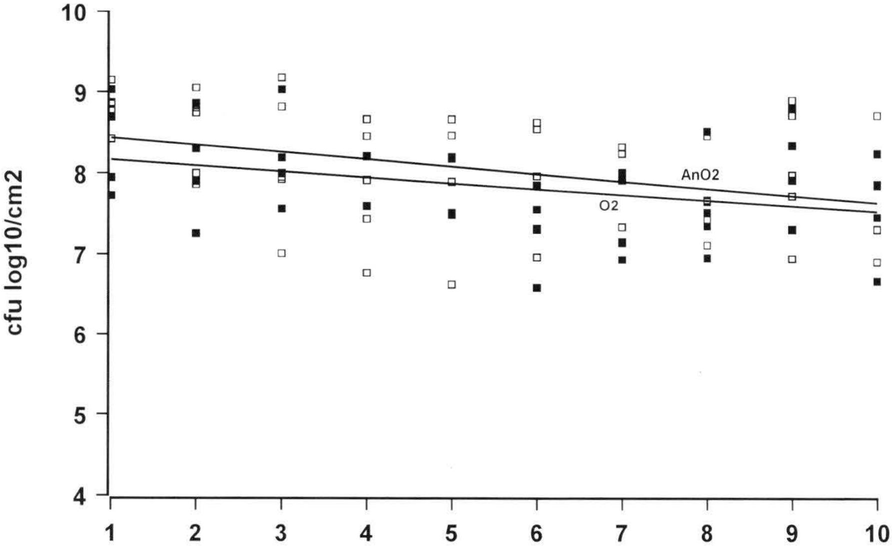


Fig. 2 : Bacterial counts from repeated sampling (n=5)

Table 4 : Bacterial counts of the microbiota from the 'back' of the tongue (7cm from the tip), from 153 toothbrush samples

	mean ± se	(range)
anaerobes n = 153	8.24 ± 0.06	(6.16-9.93)
aerobes n = 87	7.73 ± 0.07	(6.53-9.57)
aerobes:anaerobes	1:3	
microaerophiles n = 48	7.99 ± 0.12	(6.53-9.67)
Gram-negative anaerobes	7.82 ± 0.06	(4.70-9.39)
% of anaerobes	38.0	(0.003-99.99)
counts are $log_{10}cm^{-2}$		

Table 5 : Anaerobic bacterial counts divided according to odour status

odour	n	mean ± se	range
3.5	74	8.46 ± 0.08	(6.16-9.71)
2.5-3	39	8.11 ± 0.12	(6.47-9.93)
2	40	7.97 ± 0.09	(7.03-9.27)

The prevalence of oral malodour within a normal population is still hotly debated, and beyond the scope of this study. The volunteers providing samples were chosen as being atypically high or low malodorous, and therefore the possible effect of the odour status on tongue microbiota was considered. Fig. 3 shows the distribution of odour ratings for these samples and table 5 shows the anaerobic counts divided into high, low and overlap odour groups. It is apparent that the odour rating of an individual has an effect on the quantity of bacteria present on the tongue per surface area although this is a fairly loose relationship as indicated by the spread of the range for each group.

The variation in the microbiota with respect to time was assessed on 2 volunteers, from 7 separate samples taken over a period of several months (table 6). In both individuals, the variation in the counts fluctuated over the

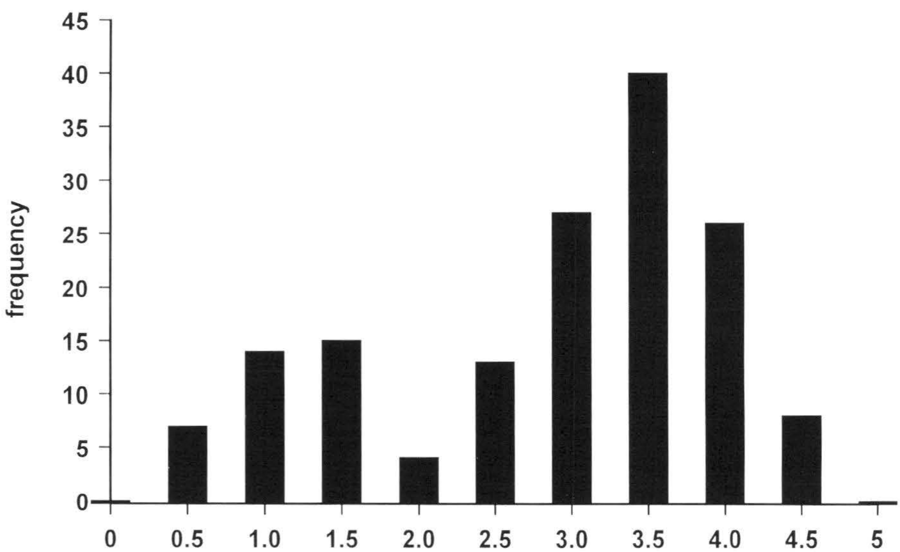


Fig. 3 : Distribution of odour ratings (n=153)

range of 1 log, considerably less than the variation between individuals. Each count appeared to vary independently so that the ratios and proportions changed.

Table 6 : Variation over time in individual's tongue microbiota

	mean ± se	range
subject 1		
anaerobes	8.65 ± 0.15	(8.18-9.18)
aerobes	8.16 ± 0.17	(7.72-8.77)
aerobes:anaerobes	1:3	
$GNanO_2$	8.26 ± 0.13	(7.70-8.66)
% of anaerobes	40.7	(28.2-82.8)
subject 2		
anaerobes	7.64 ± 0.14	(7.30-8.26)
aerobes	7.23 ± 0.08	(7.00-7.25)
aerobes:anaerobes	1:3	
$GNanO_2$	7.22 ± 0.13	(6.76-7.70)
% of anaerobes	38	(23.4-52.4)

Counts are $\log_{10}$ cm^{-2} and given as mean ± se (range)

Discussion

Very little is known about colonisation of the tongue surface. There is an increasing body of evidence to show that the tongue biofilm *per se* can be an important contributor to oral malodour but the components of this microbiota remain relatively unexamined (6).

Sampling tongue microbiota using a toothbrush is a quick, easy, reliable technique. It is a reasonably robust method although care must be taken not to move the brush laterally, nor to touch other surfaces, or to wet the brush with excess saliva. It is particularly important to be consistent in the amount of pressure applied when sampling. However, all of these points also relate to sampling with swabs.

Results using the toothbrush method illustrated that there is a significant biofilm on the tongue dorsum (1×10^7 - 1×10^9 c.f.u.), which is much greater in numerical terms than previously thought. Coykendall (7) obtained aerobic counts in the region of $2\text{-}4 \times 10^6 cm^{-2}$ from swabs. In this study the swab counts were slightly higher ($1 \times 10^7 cm^{-2}$) , but this may partly be due to the swab area being estimated. There was also considerably more variation between individuals, than within an individual, and as both Coykendall's results and

the swab results presented here were done on a small number of subjects, such differences could be due to chance. To the authors' knowledge no other work has been published quoting numbers per unit surface area.

Use of the toothbrush allows areas to be sampled further back on the tongue than are accessible by swabs. Results show that these numbers increase towards the back of the tongue, up to 5×10^9 cm^{-2}. This was not unexpected as this area suffers less abrasion during oral activities, and the crypts and fissures are much deeper, thus providing sites more conducive to the build up of a biofilm. It was surprising that a change in the nature of this biofilm could not be demonstrated, as theoretically it would be expected that such sites would harbour a slower growing, more anaerobic microbiota, with a greater odour producing potential. It must be concluded that even in the relatively thin biofilm on the front of the tongue, a substantial anaerobic environment exists.

This study has also demonstrated that a single sample does not remove all of the microbiota from that site. Despite efforts to the contrary, it is possible that some of the sequential samples were contaminated by saliva; however the implication remains, that the microbiota may be significantly more numerous (perhaps as much as tenfold greater), than reported here. Again there was no evidence of a change in the nature of the microbiota, to that of a more anaerobic balance. It may be concluded that the toothbrush bristles do not significantly penetrate crypts small enough to have an altered local environment. Perhaps a 'tailor-made' brush with fine bristles not arranged in tufts would be more appropriate.

No obvious pattern of distribution of the microbiota could be demonstrated, apart from the general increase in numbers towards the back. Thus, these findings support the use of tongue scrapings as a means of obtaining a representative sample of the microbiota.

It was interesting that this data showed only a limited increase in numbers within the biofilm when analysed with respect to malodour levels. This may, in part, be a result of the crude nature of the data, as a detailed microbiological study performed on the same subjects showed a significant increase in microbial counts associated with an increase in odour (8). It is unlikely that the differences in the microbiota seen in this study are sufficient to account for the increase in odour. Thus, future studies should focus on biochemical and physiological aspects of the microbiota in addition to quantitative/qualitative microbiological studies.

References

1. Yaegaki K. and Sanada K. (1992). Volatile sulfur compounds in mouth air from clinically healthy subjects and patients with periodontal disease. *J Periodont Res* 27:233 -238.
2. Tonzetich J. and Ng S.K. (1976). Reduction of malodor by oral cleansing procedures. *Oral Med Oral Pathol Oral Surg* 42:172-181.
3. Bosy A., Kulkarni G.V., Rosenberg M. and McCulloch C.A.G. (1994). Relationship of oral malodor to periodontitis: evidence of independence in discrete subpopulations. *J Periodontol* 65:37-46.
4. Van Winkelhoff A.J., Van der Velden U., Winkel E.G. and de Graaff J. (1986). Black-pigmented bacteroides and motile organisms on oral mucosal surfaces in individuals with and without periodontal breakdown. *J Periodont Res* 21:434-439.
5. Frisken K., Tagg J., Laws A. and Orr M. (1987). Suspected periodontopathic micro-organisms and their oral habitats in young children. *Oral Microb Immunology* 5:43-45.
6. Loesche W.J. and De Boever E.H. (1995). Main microbial contributors to oral malodor. In: Rosenberg M. (Ed) *Bad Breath: Research Perspectives.* Ramot Publishing, Tel Aviv University, Israel, pp 41-54.
7. Coykendall A.L. (1995). A method to quantitate the tongue flora. *J Dent Res* 74:127 (abstract).
8. Hartley M.G., El-Maaytah M. and Greenman J. (1995) Tongue microflora of subjects with low and high malodour levels. *J Dent Res* 74:5 87 (abstract).

Relationship of the Salivary Incubation Test with Oral Malodour Levels

*M.A. El-Maaytah, M.G. Hartley, J. Greenman, S.R. Porter and C.M. Scully**

It has been suggested that the salivary incubation test, whereby saliva is incubated at 37°C for up to 6 hours and the head-space assessed for malodour or for volatile sulphur compounds (VSC's), may reflect the microbial activities occurring in the mouth of an individual and relate to their oral malodour state. To test this hypothesis, saliva samples (n=158 from 31 individuals) were collected from subjects with varying degrees of malodour but who were otherwise healthy. Oral malodour levels were assessed by an experienced judge using a 0-5 scale at the time of saliva collection. Incubated saliva was assessed organoleptically and related to oral malodour. When samples were assessed individually a high degree of variation was noted. However, when results were pooled for each individual (3 or 4 per individual) there was a definite correlation ($p < 0.001$). The VSC levels of incubated saliva were also measured by head-space gas chromatography (GC). Although some correlation was noted between these and either incubated saliva or oral malodour, it was not as significant ($p < 0.15$). This implies that oral malodour includes components other than VSC's. Due to the high degree of variation, the salivary incubation test is only a good predictor of the oral malodour state of an individual when several salivary samples (taken over a period of time) are measured.

Introduction

Most cases of oral malodour arise from the oral cavity, as the result of bacterial activity (1). There is no ideal objective method of clinically assessing the degree of oral malodour. The simplest method is by direct sniffing of expelled mouth air (organoleptic or hedonic method) in which the intensity of oral malodour is determined on an increasing scale (2). However, the method remains subjective due to inter-judge variation, and the mental and physical state of each judge (2).

---

* M.A. El-Maaytah, M.G. Hartley, J. Greenman, S.R. Porter and C.M. Scully*, Bristol Oral Microbiology Unit, University of the West of England, Bristol, United Kingdom and *Eastman Dental Institute and Hospital, University of London, 256 Gray's Inn Road, London WC1X 8LD, United Kingdom.

Indirect measurements of oral malodour have also been used in research, based upon measuring the volatile compounds (VC) and volatile sulphur compounds (VSC) produced by putrefying salivary samples (3, 4, 5). The quantitative isolation and identification of the oral bacteria thought to be responsible for malodour (6) is another possible method which could be used to predict malodour, but it is technically difficult and slow (see also Goldberg and Rosenberg, chapter 12).

It has been suggested that saliva is one of the main sources of bad breath. Saliva contains a large reservoir of sulphur containing substrates, and studies have shown that while the salivary supernatant possesses a low potential for the formation of VSC, the sediment fraction yields values comparable to that of whole saliva (7). Salivary proteins contain sulphate (8), but are deficient in sulphur-containing amino acids (9). VSC are thought to arise from proteins via proteolysis and metabolism of the sulphur-containing amino acids by oral microbes (10, 11). Precursors include cysteine, cystine, and methionine (12, 13) found free in saliva or gingival crevicular fluid or produced as a result of proteolysis of protein substrates. Mass spectrometric and GC analyses of head-space samples identified VSC (hydrogen sulphide, methyl mercaptan and dimethyl sulphide) as the principal malodorous products of salivary putrefaction (3).

The papillated surface of the tongue provides an optimal environment for the entrapment of desquamated cells, food debris and bacterial growth capable of malodour production and a tongue coating associated with certain diseases can cause significant oral malodour (14). A tongue biofilm exists in the absence of overt disease, and is composed of epithelial cells released from the oral mucosa, micro-organisms, and leukocytes from periodontal pockets (5). Microbes within the tongue biofilm rather than those in suspension in saliva may thus play the dominant role in the generation of VSC's and hence to oral malodour (15, 5, 16).

Odour production following salivary incubation may be used as a model of malodour generation; the malodour potential of incubated saliva may reflect the oral malodour score of the subjects. This study was undertaken to determine if saliva alone or if other oral factors are important in malodour.

Methods

Subject group

Thirty-one volunteers (16 male, mean age 26.4, 15 female, mean age 24.7), were included in this study. All had good oral health and no medical complications. Subjects were asked to refrain from dietary or other activities associated with oral malodour (e.g. consumption of garlic, onion, spicy food, peppermint and the application of any fragrant products) on the day prior to and the day of the study.

Clinical assessment

The clinical assessment of each volunteer was based on a pre-prepared medical and dental questionnaire form, filled in by the clinician (NEM) during oral inspection of the subjects.

Organoleptic assessment

The trained clinician assessed the levels of oral malodour and the odour of the incubated saliva. Prior to the study, the clinician was tested for smell activity using two methods: (i) the smell identification test (SIT; Sensonic, Inc, USA), in which the judge is exposed to 40 different types of standardized aromas and is required to identify these. (ii) the smell acuity test (SAT) whereby the judge is exposed to a range of dilutions of a malodorous compound (isovaleric acid) and is required to rank them in order of the dilution. The clinician passed both tests satisfactorily.

Estimation of oral malodour

Subjects were seated in a relaxed posture and instructed to close their mouths whilst breathing normally through their nose for two minutes. The oral odour of each subject was then estimated organoleptically on the 0-5 scale, with the subjects mouth approximately 5 cm away from the judge's nose.

Salivary incubation test

A 1 ml aliquot of whole saliva was injected using a sterile disposable syringe into a sterile 20 ml glass vial with rubber sealing cap, previously filled with anaerobic gas. The vials were then incubated at 37 °C, and l00 l of head space was removed from the vial after 60 minutes using a gas-tight syringe and tested for odour organoleptically. At 30 minute intervals, further 100 l samples were taken and each tested for odour until a maximum was reached. At this point or after 6 hours, GC analysis for VSC was performed.

GC chromatography

A 1-10 l volume of headspace (37 °C) was injected onto a capillary column (HPl, 10m x 0.53mm x 2.65 m film thickness, Hewlett Packard) connected to a sulphur flame photometric detector. Helium was supplied as the carrier gas at 4psi. Temperatures were : oven 50°C, injector 250°C, detector 200°C.

Results

The incubated saliva was assessed both organoleptically and through GC analysis of VSC in the head-space. Although some correlation was noted between the salivary odour levels and the levels of VSC (Fig. 1) it was not significant ($p > 0.15$) which implies that salivary malodour includes components other than VSC.

When oral malodour levels were related to the maximum malodour levels of incubated saliva (both measured organoleptically), a high degree of variation was noted (Fig. 2) and consequently no significant correlation. However, when results (Fig. 3) were pooled for each individual (3 or 4 per individual) there was a definite correlation ($r^2 = 0.707$; $p < 0.001$).

Discussion

Our investigation indicates that the salivary incubation test could be used as a predictor of the malodour generation potential of the oral cavity of an individual although it is subject to a high degree of variation. The weak relationship (resulting in no correlation; Fig. 2) suggests that the test is only an indirect indicator of the malodour generating systems within the mouth, or

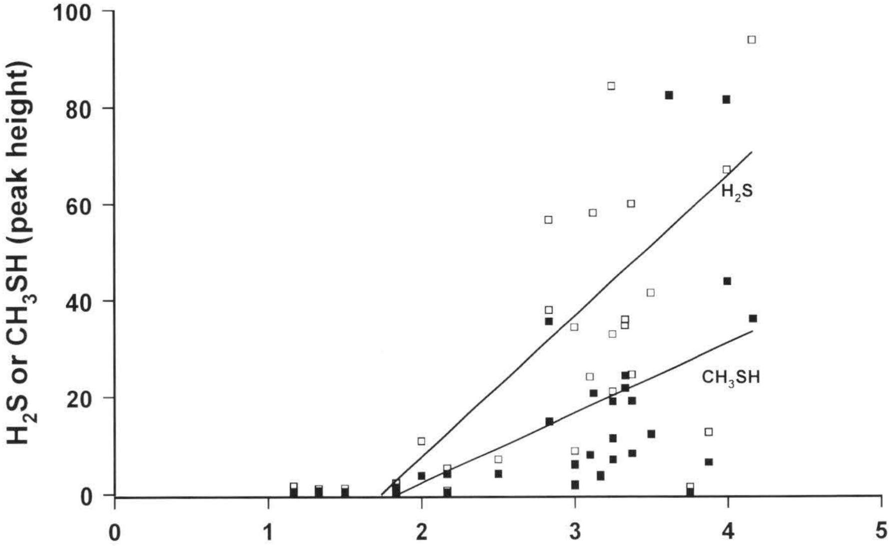


Fig. 1 : VSC vs organoleptic scores for incubated saliva.

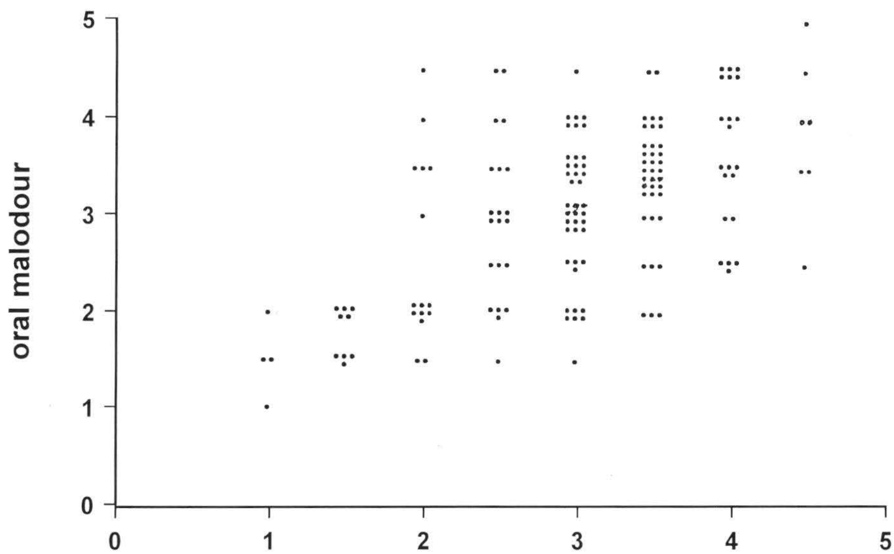


Fig. 2 : Incubated salivary odour vs oral odour (all data points).

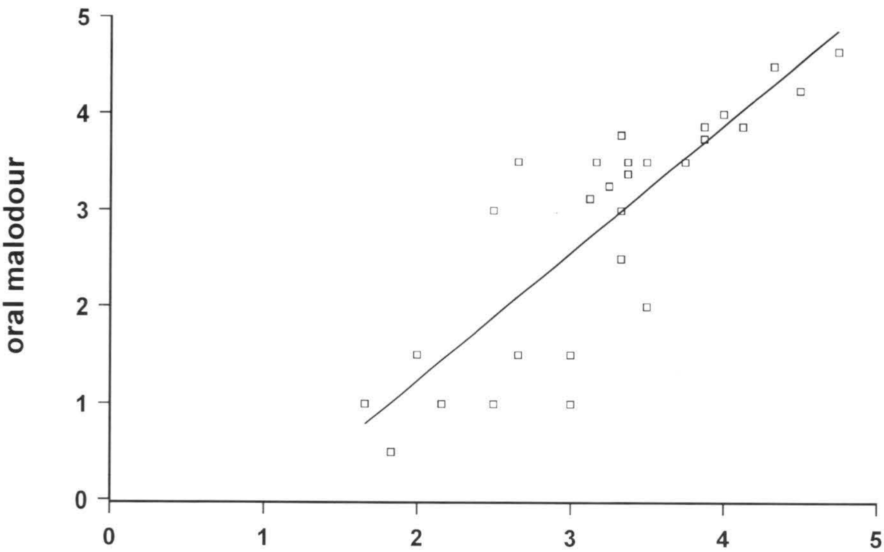


Fig. 3 : Incubated salivary odour vs oral odour.

that unknown interfering factors can be present in saliva. Thus, the salivary incubation test is only a good predictor of the oral malodour state of an individual when several salivary samples (taken over a period of time) are measured (Fig. 3).

The correlation between salivary odour and VSC in the headspace of samples is less than that which would be expected if the salivary odour was entirely due to VSC. This implies that salivary malodour (and thus probably oral malodour) includes components other than VSC's.

The role of saliva as a source of substrates for VC and VSC production in the oral cavity is still not fully understood. Once saliva is collected and incubated, a series of physicochemical shifts occur. These will not occur *in vivo* to the same extent since the mouth is more likely to reflect a 'continuous open system' rather than a batch culture. In batch conditions the mixed microbiota is subjected to batch growth conditions unlike those occurring *in vivo,* where cells grow mainly as a biofilm on the tongue. In the mouth the microbiota is subject to perturbations but the ratios of different groups one to another appears to remain relatively constant over time. The microbial flora occurring in salivary batch culture will be subjected to microbial succession whereby one group of organisms may become dominant and outgrow or out-compete the other groups. In particular, strictly anaerobic organisms may be inhibited

by oxygen, and out-competed by faster growing facultative or oxygen indifferent types such as the streptococci. These factors may explain the lack of strong correlation between salivary incubation and oral malodour.

References

1. Loesche W.J. and De Boever E.H. (1995) Strategies to identify the main microbial contribution to oral malodour. In : Rosenberg M. (Ed.) *Bad breath: research perspectives.* Ramot Publishing, Tel Aviv University, Israel, pp. 41-54.
2. Rosenberg M. and McCulloch C.A.G. (1992) Measurement of oral malodour: Current method and future prospects. *J Periodontol* 63:776-782.
3. Tonzetich J. (1977) Production and origin of oral malodour : A review of mechanisms and methods of analysis. *J Periodontol* 48:13-20.
4. Kostelc J.G., Preti G., Zelson P.R., Brauner L. and Baehni P. (1984) Oral odours in early experimental gingivitis. *J Periodont Res* 19:303-312.
5. Yaegaki K. and Sanada K. (1992) Biochemical and clinical factors influencing oral malodour in periodontal patients. *J Periodontol* 63:783-789.
6. Hartley G., El-Maaytah M. and Greenman J. (1995) Tongue microflora of subjects with low and high malodour levels. *J Dent Res* (Special Issue), 74, 587, Abstr 1491.
7. Tonzetich J. and Kestenbaum R.C. (1969) Odor production by human salivary fraction and plaque. *Archs oral Biol* 14:815-827.
8. Kolendo A.B. (1970) Sulphated groups in human salivary proteins : A comparative histochemical and chemical study. *Archs oral Biol* 15:323-333.
9. Leach S.A., Critchley P., Kolendo A.B. and Saxton C.A. (1967) Salivary glycoproteins as components of the enamel integuments. *Caries Res* 1:104-111.
10. Prinz H. (1930) Offensive breath, its causes and prevention. *Dental Cosmos* 72:700-707.
11. Richter V.J. and Tonzetich J. (1964) The application of instrumental technique for the evaluation of odouriferous volatiles from saliva and breath. *Archs oral Biol* 9:47-53.
12. Tonzetich J. and Johnson P.W. (1977) Chemical analysis of thiol, disulphide and total sulphur content of human saliva. *Archs oral Biol* 22:125-131.

13. Yaegaki K. (1989) Periodontal disease and precursor of oral malodour component. *J Dent Health* 39:733-741
14. McDowell J.D. and Kassenbaum D.K. (1993) Diagnosis and treating halitosis. *J Am Dent Ass*, 124:55-64.
15. Kaizu T. (1976) Analysis of volatile sulphur compounds in mouth air by gas chromatography. *J Jpn Assoc Periodontol* 18:1-12.
16. Bosy A., Kulkarni G.V., Rosenberg M. and McCulloch C.A.G. (1994) Relationship of oral malodor to periodontitis : Evidence of independence in discrete subpopulations. *J Periodontol* 65:37-46.

Production of Oral Malodour in an *in vitro* System

*S. Goldberg and M. Rosenberg**

Most cases of bad breath (halitosis, fetor oris) are considered to be the result of production of malodorous compounds by oral bacteria (1-3). Since the oral cavity is colonized by some 500 species of bacteria, identification of those responsible for malodour is a difficult process. The tongue, which is suspected to be one of the main sources of oral malodour, accomodates a diverse microbiota which remains largely uncharted. Gordon and Gibbons (4) studied the tongue microbiota of six subjects and identified several malodorous bacteria (*Bacteroides, Fusobacteria, Peptostreptococcus*) as prominent tongue microbiota. McNamara *et al.* (3) showed that among 14 oral isolates only the Gram-negative bacteria produced malodour. Solis-Gaffar *et al.* (5) subsequently showed that among 12 species, only those which were Gram-negative produced volatile sulphur compounds (VSC). Slots (6) found that *Fusobacterium nucleatum* was prominent in the microbiota of patients with gingivitis or periodontitis. This species is able to produce high levels of VSC, which contribute to bad breath (7) In 1981, Tonzetich and McBride (8) showed that pathogenic strains of Bacteroides produced higher levels of VSC than non pathogenic bacteroides. Persson and coworkers (7) described a wide range of bacterial species capable of producing hydrogen or methylmercaptan from serum. Kleinberg and Codipilly (9) recently reported odour formation by various bacterial species over time as a function of amino acid addition. A list of potentially malodorous oral bacteria is shown in Table 1.

Table 1 : Some oral bacteria that have been implicated in oral malodour

Prevotella intermedia (9, 7)
Fusobacterium nucleatum (9, 7, 10)
Porphyromonas gingivalis (9, 7)
Porphyromonas endodontalis (11)
Prevotella loeschii (11, 7)
Hemophilus parainfluenzae (?) (9)
Treponema denticola (7)

* S. Goldberg and M. Rosenberg, The Maurice and Gabriela Goldschleger School of Dental Medicine and Department of Human Microbiology, Sackler Faculty of Medicine, Tel-Aviv University, Ramat-Aviv 69978, Tel-Aviv, Israel.

Veillonella species (9, 10)
Peptostreptococcus anaerobius (7);
Micros prevotii (7);
Centipeda periodontii (7);
Selemonad aremidis (7);
Eubacterium species (7);
Bacteroides species (7);
Fusobacterium periodonticum (7)
Prevotella melaninogenica (10)
Klebsiella pneumoniae (10)
Enterobacter cloacae (10)
Citrobacter species (Goldberg *et al.*, unpublished.)
Stomatococcus mucilaginus (12)

An *In vitro* System

In order to evaluate the microbial physiology of oral malodour production, and to investigate the gases responsible for this phenomenon, we sought a reproducible *in vitro* system which yields "bad breath-like" odour in a test tube. Samples of saliva, tongue scrapings or paper points, inserted into periodontal pockets, were inoculated into different growth media. After 72 hours of incubation at 37°C the tubes were scored by an odour judge and volatile sulphides were concomitantly measured using the model 1170 (Interscan Corp. USA) sulphide monitor. Results are summarized in Table 2. In all cases, decarboxylase medium (containing peptone, yeast extract and dextrose, as well as a pH indicator, bromcresol purple) gave an odour most resembling bad breath. No correlation was evident between volatile sulphide levels and odour resembling bad breath. For example, whereas incubation in brain heart infusion broth and Schaedler broth resulted in copious VSC production, neither headspace resembled the smell of oral malodour. Similarly, although incubation in saline alone gave an odour somewhat similar to oral malodour, only a low level of VSC was observed.

Table 2 : In vitro production of malodour following 72h incubation[a]

Medium/Buffer	Qualitative organoleptic assessment[b]	Volatile sulphide (ppb)[c]
Brain Heart Infusion	not typical	1600
Decarboxylase	typical	1250
Schaedler	not typical	1600
PBS	not typical	40
Carbonate buffer (pH 8)	not typical	5
Salin	somewhat typical	50

[a] *Morning saliva was inoculated 1:25 into medium/buffer and incubated for 72 hours at 37°C.*
[b] *Odour was determined by an odour judge.*
[c] *Volatile sulphide was determined using the Interscan 1170 portable sulphide monitor (Interscan Corp. Chatsworth, CA), fitted with a bent plastic straw which was inserted 2 cm into the test tube.*

Influence of Amino Acid Addition on Odour Parameters

The influence of different amino acids on odour production in the decarboxylase model system was examined by adding 0.5% amino acid to the decarboxylase medium. Saliva was added to the medium (1:25) and following 72 hours incubation, odour judge scores, VSC level and pH were measured. Results are presented in Table 3. Among the amino acids added, only methionine and proline elevated the odour present in decarboxylase medium alone. Interestingly, VSC levels were elevated in the presence of additional lysine and histidine. Aspartic and glutamic acids inhibited odour production, presumably by lowering the pH. The finding that malodour production was stimulated in the presence of proline contrasts with the results of the study of Kleinberg and Codipilly (9), who measured malodour production by specific species, in the presence of various amino acids. In their study, cysteine, ornithine, methionine, tryptophan and cystine stimulated malodour to a much greater extent than other amino acids.

Table 3 : Effect of amino acid on malodour-associated parameters[a]

Amino Acid added[b]	Organoleptic score[c]	pH following incubation	Volatile Sulphide (ppb)[d]
no addition	4	5.0	380
methionine	5	5.0	> 1000
proline	5	5.0	270
lysine	4	8.0	> 1000
ornithine	4[e]	7.5	950
threonine	4[e]	5.0	260
histidine	4[e]	7.5	> 1000
alanine	4[e]	5.0	120
phenylalanine	4[e]	5.0	140
tyrosine	4	5.0	80
aspatic acid	1	3.5	10
glutamic acid	1	4.0	10

[a] *Morning saliva was inoculated 1:25 into decarboxylase medium with added amino acids, as indicated*
[b] *5 mg/ml*
[c] *On a scale of 0-5*
[d] *Volatile sulphide was determined using the Interscan 1170, fitted with a bent plastic straw which was inserted 2 cm into the test tube.*
[e] *Not typical*

Modification of decarboxylase medium

In an attempt to identify the components in the decarboxylase medium which contribute to oral malodour, various modifications were made. Results are shown in Table 4. With the exception of glucose, all the nutrients present in the decarboxylase medium appeared to contribute to malodour production. Dilution of the medium (1:4) similarly resulted in loss of odour.

Table 4 : Effect of modifications in decarboxylase medium on malodour production[a]

Modification	Organoleptic Assessment
None	typical
Without peptone	no odour
Without glucose	typical after 48 hours but not after 72 hours
Peptone only	not typical
Yeast extract only	not typical
Casamino acid with added lysine (5mg/ml)	no odour
Decarboxylase medium diluted 1:4	no odour

[a] *Unmodified decarboxylase medium consisted of 0.5% peptone, 0.3% yeast extract, 0.1% glucose and 0.0002% bromcresol purple*

Isolation of odourigenic bacteria

A series of attempts was made to isolate individual odour-producing colonies from the decarboxylase medium following outgrowth, using various types of solid media and under both aerobic and anaerobic growth conditions. The only bacteria that produced a foul odour bearing any resemblance to bad-breath were isolates of the family *Enterobacteriaceae*, mainly *Klebsiella pneumoniae*, *Klebsiella oxytoca* and *Enterobacter cloacae*.

Table 5 : Prevalence of Enterobacteriaceae in three subject populations

Subject Group	Number of Subjects	Prevalence
Orthodontic patients	107	13%
Subjects complaining of oral maladour	74	27%
Denture patients	45	48%

Although *Klebsiella pneumoniae* was included by Solis-Gaffar and coworkers (5) as a predominant oral species and was shown in their study to produce malodour, *Enterobacteriaceae* are not considered to be common residents of the human mouth. Sedgley and Samaranayake (13) have summarized 16 studies in which the oral prevalence of *Enterobacteriaceae* and other Gram negative rods was determined. In most studies, not more than 24% of the subjects tested were found to carry such microorganisms. Only studies conducted in the Far East (Malaysia and Hong Kong) yielded higher prevalence scores.

In an attempt to determine whether *Enterobacteriaceae* might be involved in bad breath production *in vivo*, we started to look for the presence of these bacteria in different population groups in Israel. Three population groups were studied: (i) orthodontic patients; (ii) subjects presenting at an oral malodour clinic; and (iii) denture wearers. The prevalence of *Klebsiella, Enterobacter, Citrobacter* and *Serratia* was determined by taking samples from interdental, tongue, or denture palate and inoculating them onto MacConkey and citrate agar media. Results are shown in Table 5. The highest prevalence of *Enterobacteriaceae* was found in the denture wearers population (48%). The orthodontic patients, who were mostly teenagers, had the lowest prevalence of *Enterobacteriaceae*.

When the presence of the above-mentioned bacteria was compared to organoleptic levels of oral malodour in the above subjects, no correlations were observed. Nevertheless, many of these isolates produced "bad breath-like" odour *in vitro* when inoculated into decarboxylase medium.

The high prevalence of *Klebsiella* and *Enterobacter* in the population of denture wearers indicate that these bacteria might play a role in denture halitosis, a common problem among elderly people. Preliminary *in vitro* experiments showed production of typical denture-type odour by these bacteria when incubated on dentures.

Other Factors Influencing Odour Production

Saliva inoculation in decarboxylase medium results in production of odour resembling bad breath, after three days incubation. Incubation of saliva inoculated into decarboxylase medium with ensuing odour production could be performed anaerobically or semi-aerobically (without shaking). Aerobic incubation, with shaking, eliminated the odour but apparently did not harm the responsible microorganisms: when tubes, shaken for 24 hours were subsequently incubated under anaerobic conditions, odour was produced. Incubation of samples from the back of the tongue or from deep periodontal pockets, in decarboxylase medium, also resulted in production of odour resembling bad breath. Diluting the sample (whether obtained from saliva, periodontal pockets or tongue scraping) eliminated the production of malodour when the dilutions were beyond 1:250. Diluting the medium 1:4, addition of 0.2µg/ml gentian violet, 0.2M NaCl, 20µg/ml ampicillin, 2µg/ml penicillin, 0.8mg/ml lysozyme, 10µg/ml potasium tellurite or 80µg/ml metronidazole, all resulted in elimination of the odour production. Growth at 42°C or at a pH lower than 6 also resulted in elimination of the odour.

Discussion

The identification of specific microorganisms which are actually involved in oral malodour production is problematic. Many oral species produce odour *in vitro* (9, 7), however, it is not known whether this reflects their in vivo activity. Furthermore, oral malodours have characteristic qualities which are usually dissimilar to the odours of headspace of axenic cultures of oral microorganisms. In order to study this problem, we devised a simple procedure that allows for production of bad breath-like odour (more specifically, an odour similar to that of subgingival plaque) in the test tube. In this technique, oral samples (saliva, tongue scrapings, or samples taken by paper points from periodontal pockets) are inoculated into decarboxylase medium and incubated for 72h at 37°C. Odour production can be modulated by supplementing the decarboxylase medium with individual amino acids. Addition of methionine and proline resulted in strongest odour production, as determined by organoleptic determination. Addition of lysine, histidine, ornithine and methionine were most active in stimulating production of volatile sulphides.

Following incubation, samples were plated out and odourigenic colonies identified. In many cases, isolates were members of the Enterobacteriaceae. These microorganisms were most prevalent among denture wearers, as compared with other populations. No correlation, however, was found between prevalence of enterobacterial species and malodour levels.

Malodour production was inhibited by certain antibiotics, low pH, antimicrobial agents, and incubation at high temperatures. Significantly, although odour was not produced during aerobic incubation, subsequent transfer of tubes to anaerobic conditions resulted in elaboration of typical malodour. Further work is currently underway to test the potential contribution of *Enterobacteriaceae* to denture malodour.

Acknowledgements

We thank Y. Mazor for excellent technical assistance.

References

1. Howe J.W. (1898) The breath, and the diseases which give it a fetid odor. 4th Edition, New York: D. Appleton and Co.
2. Berg M. and Fosdick L.S. (1946) Studies in periodontal disease. II.

Putrefactive organisms in the mouth. *J Dent Res* 25:73-81.
3. McNamara T.F., Alexander J.F. and Lee M. (1972) The role of microorganisms in the production of oral malodor. *Oral Surg* 34:41-48.
4. Gordon D.F. and Gibbons R.J. (1966) Studies of the predominant cultivable micro-organisms from the human tongue. *Archs oral Biol* 11:627-32.
5. Solis-Gaffar M., Fischer T. and Gaffar A. (1979) Instrumental evaluation of odor produced by specific oral microorganisms. *J Soc Cosmet Chem* 30: 241-242.
6. Slots J. (1977) The predominent cultivable microflora of advanced periodontitis. *Scand J Dent Res* 85:114-121.
7. Persson S., Edlund M.B., Claesson R. and Carlsson J. (1990) The formation of hydrogen sulfide and methylmercaptan by oral bacteria. *Oral Microbiol Immunol* 5:195-201.
8. Tonzetich J. and McBride B.C. (1981) Characterization of volatile sulphur production by pathogenic and non pathogenic strains of oral bacteroides. *Archs oral Biol* 26: 963-969.
9. Kleinberg I. and Codipilly M. (1995) The biological basis of oral malodor formation. In : Rosenberg M. (Ed) *Bad Breath: Research Perspectives. Ramot Publishing, Tel Aviv University, Tel-Aviv, Israel, pp 13-39.*
10. Niles H. and Gaffar A. (1995) Advances in mouth odor research. In Rosenberg M. (Ed) *Bad Breath: Research Perspectives.* Ramot Publishing, Tel Aviv University, Tel-Aviv, Israel, pp 55-69
11. Loesche W.J. and De Boever E.H. (1995) Strategies to identify the main microbial contributors to oral malodor. In : Rosenberg M. (Ed*) Bad Breath: Research Perspectives.* Ramot Publishing, Tel Aviv University, Tel-Aviv, Israel, pp 41-54.
12. Greenman J., El-Maaytah M.A., Hartley M.G. and McAloon S. (1996) Proteolytic activity of *Stomatococcus mucilaginus.* In : van Steenberghe D. and Rosenberg M. (Eds) *Bad Breath: a multidisciplinary approach.* Leuven University Press, Leuven, Belgium, chapter 14, pp 157-162.
13. Sedgley C.M. and Samaranayake L.P. (1994) The oral prevalence of aerobic and facultatively anaerobic gram-negative rods and yeasts in Hong Kong Chinese. *Archs oral Biol* 39:459-466.

A Microbiological Method for the Detection of Hydrogen Sulphide Producing Oral Spirochetes Associated with Periodontitis

A. De Ciccio, L.Z.G. Touyz, M.D. Rahal, A. Klitorinos and E.C.S. Chan[*]

Breakdown of tissues with sulphur-containing essential amino acids (cysteine, cystine, methionine) by sulfhydrase-positive (S.ase+) bacteria yields volatile sulphur compounds (VSCs, e.g., SO_2, H_2S, CH_3SH) as a major part (90%) of substances redolent in oral malodour. Many invasive pathogenic bacteria are S.ase+, but reports on S.ase+ spirochetes associated with periodontitis (SAWPs) are rare. Detection of VSCs with a portable sulphur monitor (Halimeter, Interscan Corp., Chatsworth CA, USA), gas chromatography, or mass spectrometry does not focus on biologic bacterial sulfhydrase activity. The conventional method for detecting H_2S microbiologically uses ferric ammonium citrate (FAC) in the growth medium, which blackens to iron sulphide salt clusters with S.ase+ colonies or along a S.ase+ inoculum stab line. Because citrate chelates minerals, six other iron salts were also tested. By combining ferrous ammonium sulfate (FAS) and New Oral Spirochete (NOS) medium gelled with agarose, the best positive demonstration for H_2S production by stock cultures of S.ase+ spirochetes was obtained. Subgingival bacterial plaques from diseased periodontal pockets with 7-10 mm probing depths, were dispersed serially in NOS-agarose FAS medium with rifampicin. This yielded similar black SAWP-colonies indicating potent sulfhydrase and VSC production. This vivid demonstration provides a successful method to demonstrate that SAWP's are major contributors to oral malodour from gingivitis. Not all SAWPs were S.ase+. Further ongoing research will refine prevalences, possible SAWP subtypes and other associations with clinical manifestations.

Introduction

Breakdown of all organic matter containing sulphurous amino acids (cysteine, cystine, methionine) by sulfhydrase-positive (S.ase+) bacteria yields volatile sulphur compounds (VSC), such as hydrogen sulphide (H_2S) and methyl

---

* A. De Ciccio, L.Z.G. Touyz, M.D. Radal, A. Klitorinos and E.C.S. Chan, Department of Microbiology and Immunology, Faculty of Dentistry, McGill University, 3775 University Street, Montreal PQ Canada H3A 2B4.

mercaptan (CH_3SH). These two compounds account for 90% of total VSC redolent in oral malodour (1). VSC production is accentuated in tissue degenerative conditions such as gingivitis, periodontitis, and necrotic gingivitis (2). VSC detection is conventionally determined by a portable sulphide monitor (Halimeter), gas chromatography, or mass spectrometry but such methods do not focus on microbiological reactions or microbial ecologic sites.

Spirochete species of the genus *Treponema* are commonly found in subgingival plaque of periodontal pockets (3) and correlate with progression of periodontitis (4). Recently, bacterial culture media have been used for detection of many oral odour-producing bacterial species other than spirochetes (5, 6), but to date, no medium has been described for identifying S.ase+ spirochetes associated with periodontitis. This report describes a microbiological method for detection of hydrogen sulphide-producing oral spirochetes associated with periodontitis.

Materials and methods

Conventional detection of hydrogen sulphide production by bacteria is through incorporating ferrous or ferric salts in the growth medium. This allows black iron sulphide salt clusters to form within S.ase+ colonies or along an inoculum stab line after growth of S.ase+ bacteria (7). Since oral spirochetes are nutritionally fastidious, the basal medium used to grow spirochetes was the NOS agarose medium (8), but it was modified for use to detect hydrogen sulphide production. Ferric ammonium citrate (0.05%, FAC) was incorporated into NOS agarose medium to detect H_2S production upon growth of oral anaerobic spirochetes (OAS). The spirochetes used were American Type Culture Collection (ATCC) strains maintained in the oral microbiology laboratory at McGill University.

While the use of FAC permitted identification of S.ase+ strains, the inherent toxicity of FAC on spirochetes is ill-defined, since citrate is known to have chelating or sequestering properties on many trace metals. Therefore, a comparison of various selected iron salts (at 0.05% final concentration in NOS agarose medium), namely, FAC; ferric chloride, ferric oxalate, ferric sulfate, ferrous sulfate, and ferrous ammonium sulfate (FAS), was undertaken to determine their comparative toxicity for OAS. Toxicity was evaluated by recovery of colony-forming units (CFU) in NOS agarose medium inoculated from 10-fold serial dilutions of pure cultures of OAS in NOS broth.

Preliminary experiments were carried out to determine the contribution of OAS in periodontal pockets to oral malodour on the basis of H_2S production.

Two sterile paper points were inserted into diseased periodontal pockets with 7-10 mm probing depths. These points were suspended in 2 ml reduced transport medium (9) and vortexed vigorously to dislodge adherent OAS. One ml of the suspension was inoculated into a 9-ml NOS broth tube and further 10-fold serial dilutions were carried out with NOS broth. One ml of each dilution was then inoculated into 29 ml molten (37°C) NOS agarose medium (containing 2µg/ml of the antibiotic rifampicin as well as 0.05% FAS in a tissue culture flask as described previously (10)).

Results

The incorporation of FAC into NOS agarose medium showed blackening along the stab line when inoculated with pure cultures of *T. denticola* ATCC 35405 and ATCC 35404 as shown in Fig. 1 (Note that a culture of the putative

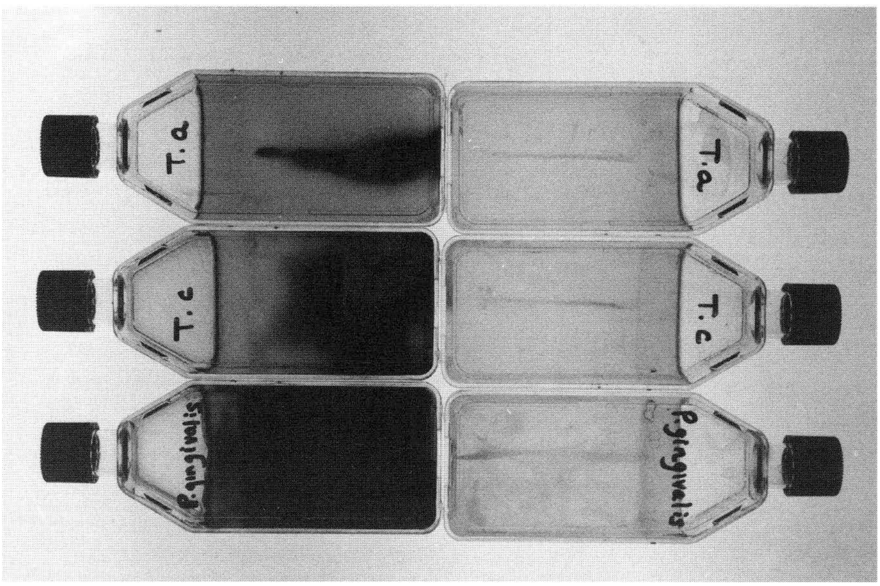


Fig. 1 : Stabs of Treponema denticola ATCC 35405 (labelled as T.a.), T. denticola ATCC 35404 (labelled as T.c.), and Porphyromonas gingivalis in NOS agarose medium (right) and with ferric ammonium citrate (left). Blackening along the stab line and in the surrounding medium indicates H_2S production and that the spirochete species as well as P. gingivalis are S.ase+.

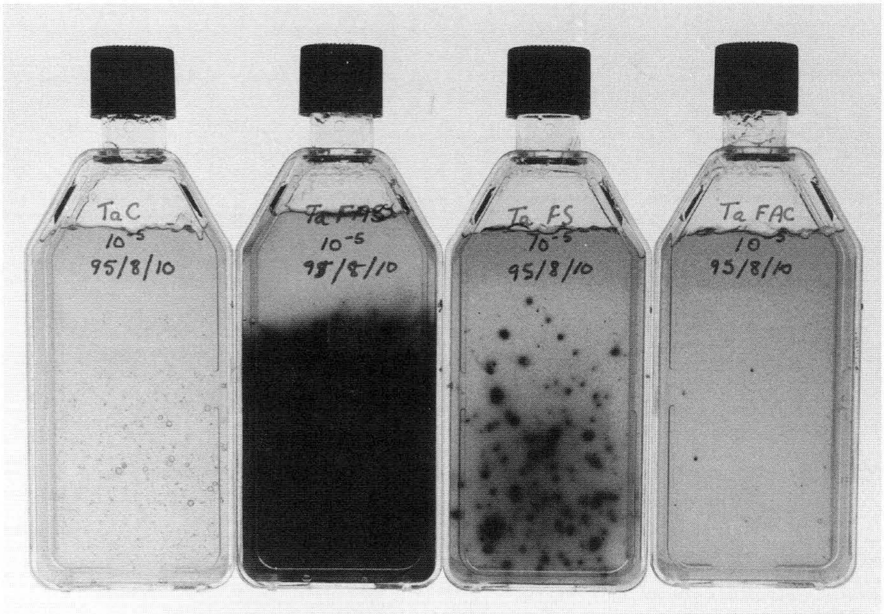


Fig. 2 : Comparative CFU recovery of a 10^{-5} dilution of T. denticola ATCC 35405 in the presence of various iron salts. Left to right : control NOS agarose medium (numerous colorless colonies with no blackening); with ferrous ammonium sulfate added (highest number of black colonies; too numerous to be discerned as discrete colonies in this dilution); with ferrous sulfate added (numerous black colonies); with ferric ammonium citrate added (few black colonies).

periopathogen *Porphyromonas gingivalis*, used for comparison, also produced abundant blackening of the medium.). Similar results were obtained with *T. socranskii* ATCC 35536, and *T. vincentii* ATCC 35580.

A comparison of various iron salts added at 0.05% final concentration to NOS agarose medium revealed that FAC, ferric chloride, ferric oxalate and ferric sulfate were highly toxic to OAS since CFU recovery using *T. denticola* ATCC 35405 was nil or extremely low. However, ferrous sulfate was partially inhibitory and slightly reduced the CFU count while FAS was the least toxic (about 50% CFU recovery compared to control NOS agarose medium without iron additive) leading to the highest counts of all the iron salt additives tested. These results are illustrated in Fig. 2. FAS was the iron salt of choice in all subsequent experiments.

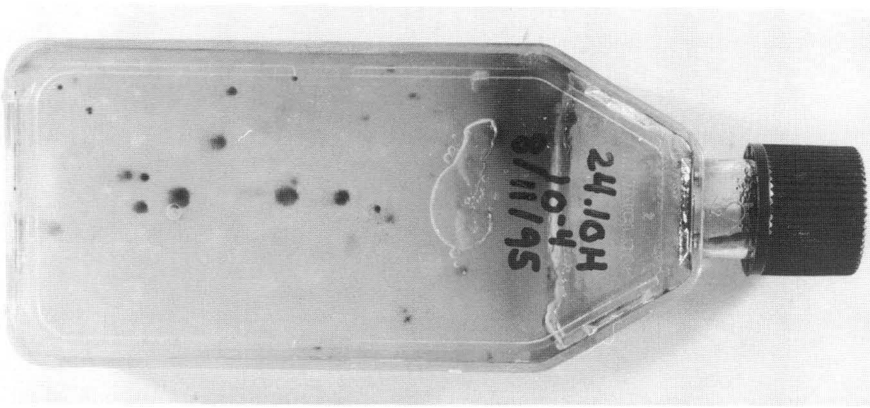


Fig. 3 : CFU recovery of a 10^{-4} dilution of a sample from a periodontal pocket using the method described in the text. Black (H_2S-producing) and colorless colonies are visible.

Fig. 3 shows the use of NOS agarose medium containing rifampicin and FAS in detecting H_2S-producing colonies of OAS from a sample derived from paper points used in sampling; black OAS colonies are clearly visible.

Discussion

A practical and convenient method has been developed for the detection and enumeration of H_2S-producing OAS from deep periodontal pockets by a modification of an established technique for recovery of spirochetes (8). FAS was chosen as the precipitating iron salt in the presence of H_2S since it was the least toxic of several iron salts tested for a pure culture of *T. denticola* ATCC 35405. Preliminary experiments with paper point samples from periodontal pockets have shown that FAS may not be toxic for all spirochetes and may even be stimulatory.

The use of rifampicin decreases the interference of CFUs of other types of *Eubacteria* from the pocket and facilitates the counting of OAS colonies (both S.ase+ and S.ase-). The recovery of H_2S-producing spirochete CFUs in preliminary experiments have shown that OAS are major contributors to oral malodour from periodontitis. Ongoing research with significant statistical samplings will reveal prevalences and occurrences of S.ase+ spirochetes in the etiology and establishment of the disease state. Possible spirochete types or strains may also be correlated with clinical findings.

References

1. Persson S., Edlund M-B., Claesson R. and Carlsson J. (1990) The formation of hydrogen sulfide and methyl mercaptan by oral bacteria. *Oral Microbiol Immunol* 5:195-201.
2. Tonzetich J. (1977) Production and origin of oral malodor: a review of mechanisms and methods of analysis. *J Periodontol* 48:13-19.
3. Cheng S-L. and Chan E.C.S. (1983) The routine isolation, growth, and maintenance of the intermediate-size oral anaerobic spirochetes from periodontal pockets. *J Periodont Res* 18:362-368.
4. Simonson L.G., Goodman C.H., Bial J.J. and Morton H.E. (1988) Quantitative relationship of *Treponema denticola* to severity of periodontal disease. *Infect Immun* 56:726-728.
5. Van der Hoeven J.S. and Schaeken M.J.M. (1995) Sulfate-reducing bacteria in the periodontal pocket. *J Dent Res* 74:587 (Abstract).
6. Turng B., Minah G. and Zhang M. (1995) Evaluation of bacterial culture media for oral odor-producing species. *J Dent Res* 74:587 (Abstract).
7. Gerhardt P., Murray R.G.E., Wood W.A. and Krieg N.R. (Ed) (1994) Methods for general and mocular bacteriology. Washington DC, American Society for Microbiology.
8. Chan E.C.S., Siboo, R., Touyz, L.Z.G., Qiu Y-S. and Klitorinos A. (1993) A successful method for quantifying viable oral anaerobic spirochetes. *Oral Microbiol Immunol* 8:80-83.
9. Holdeman, L.V., Cato E.P. and Moore W.E.C. (Ed) (1977) Anaerobe Laboratory Manual. 4th ed., Blacksburg, VA, Virginia Polytechnic Institute and State University.
10. Qiu, Y-S., Klitorinos, A., Rahal, M.D., Siboo, R. and Chan, E.C.S. (1994) Enumeration of viable oral spirochetes from periodontal pockets. *Oral Microbiol Immunol* 9:301-304.

Proteolytic Activity of *Stomatococcus mucilaginus*

*J. Greenman, M.A. El-Maaytah, M.G. Hartley and C. McKenzie**

Stomatococcus mucilaginus is commonly isolated (isolation frequency, 52%) from the dorsum of the tongue in healthy subjects. Levels may be as high as 6.6 x 10^7 c.f.u./cm^2. This aerobic, Gram positive coccus is not particularly malodorous when grown on plates, but many strains are proteolytic and they represent the highest proportion of N-α-benzoyl-L-arginine-p-nitroanilide (BAPNA) positive species found on the tongue. Strains, isolated from individuals with low to middle malodour, was inoculated into brain heart infusion broth and incubated with aeration at 37°C for 24h. Cells and supernatant were separated by centrifugation and proteolytic activity measured by spectrophotometric assay using BAPNA. Culture supernatant was concentrated 20-fold by ultrafiltration and samples were subjected to gel perfusion chromatography. Active fractions were pooled. Results from assays showed that the proteolytic activity was almost exclusively extracellular with a pH optimum for catalytic activity of pH 9.5. The active fraction was relatively heat stable at 80°C (half-life of 0.6h at pH 9.5) and had a molecular weight of 44,000 Da. The enzyme was strongly inhibited by leupeptin but, in contrast to the trypsin-like protease of *Porphyromonas* did not require reducing conditions for activity. *In vivo,* extracellular alkaline protease from *Stomatococcus* may play an important role in the hydrolysis of proteinaceous substrates on the tongue and may help supply nutrients to other members of the tongue biofilm community, including species which are capable of generating malodour.

Introduction

Stomatococcus and related species are Gram positive, oxidase negative, VP positive, aerobic/facultatively anaerobic, catalase positive, cocci (1). In our laboratories (2) they are commonly isolated from the dorsum of the tongue in healthy subjects (isolation frequency, 52%). Levels may be as high as 6.6 x 10^7 c.f.u./cm^2 which may represent a significant proportion of the total microbiota from the tongue surface. Many strains are positive for trypsin-like

---

* J. Greenman, M.A. El-Maaytah, M.G. Hartley and C. McKenzie, Bristol Oral Microbiology Unit, University of the West of England, Bristol, United Kingdom.

protease when tested using N-α-benzoyl-L-arginine-p-nitroanilide (BAPNA). The proteolytic activity is of interest since it may represent an important step in the conversion of soluble and insoluble proteins on the tongue surface into key substrates (cysteine, cystine, methionine, arginine, lysine, tryptophan) which may then be used by the tongue biofilm community to produce malodorous compounds. This preliminary study reports on the production and some of the properties of this trypsin-like activity.

Methods and materials

Bacterial strains : Strain D7 was isolated from the dorsum of the tongue from an individual with low malodour. Strains A1 and D5 were isolated from individuals with low to middle levels of malodour. These were maintained on fastidious anaerobe agar (FAA) supplemented with horse blood (5% v/v). Plates were incubated aerobically at 37°C. Cultures were stored at -70°C on porous beads (Microbank).

Culture : Strains were grown in batch culture using four different media: (i) BHI (brain heart infusion broth, 3.7% w/v: Difco) (ii) TYE (tryptone, 1% w/v plus yeast extract, 0.5% w/v; Oxoid) (ill) TYE + glucose (0.2%w/v) (iv) TYE + casein (0.2% w/v; BDH). Broths were prepared as 100 ml volumes in 250 ml flasks. Following inoculation, flasks were incubated with aeration (rotary incubator) at 37°C for 24-48 h. Cells and supernatant were separated by centrifugation (4,000g; 30 min) and tested for proteolytic activity.

Protease assay: The assay consisted of 1.0 ml enzyme preparation (supernatant, resuspended cells or active fractions), 1.8 ml of 0.1M zwitterionic buffer (see table 1) and 0.2 ml solution of 0.2mM BAPNA (Sigma). The absorbance change was measured spectrophotometrically at 410 nm.

Table 1 : Zwitterionic buffers used in pH activity experiments

Buffer	Abbreviation	pH range
N-2-hydroxyethylpiperazine-N'-2-ethaneulphonic acid	HEPES	6.8-8.2
N,N-bis-[2-hydroxyethyl]-glycine	BICINE	7.6-9.0
2-[N-cyclohexylamino]ethanesulphonic acid	CHES	8.9-10.0

The pH of each assay mixture was checked before and after incubation to ensure that no pH change had occurred (19°C ± 2°C)

Units of enzyme activity: A unit of activity (U) is defined as nmol product (formed under the conditions of the assay) per hour. Specific activity is defined as enzyme units per mg dry wt of bacterial cells. Specific activity in the supernatant fraction was related to the dry weight of cells in the culture sample before centrifugation. The quantity of product formed was calculated using the molar extinction coefficient () of *p*-nitroaniline at 410 nm (10,000 M^{-1} cm^{-1}). Dry weights were determined by the method described by Holland *et al.* (3) and culture absorbances were related to dry weight with reference to a calibration curve.

Concentration and characterization : Culture supernatant was concentrated 20-fold by ultrafiltration at 4°C using a 1O kDa filter (PM10, 76mm diameter, Amicon) and samples were subjected to gel perfusion chromatography (Superose 12 10/30) equilibrated with buffer (50mM Tris, 100mM NaCL, pH7.4). Fractions were assayed for TLPase and active fractions pooled.

A range of protease inhibitors and metal ions were tested for their effect on the crude enzyme. The compounds used are listed in table 2. The enzyme was tested for heat stability as follows: A 10 ml sample of crude enzyme in a universal bottle was incubated in a water bath at 80°C. At time zero and at various time intervals, 1 ml aliquots were removed and assayed for TLPase using the assay described. The rate of denaturation was calculated from a plot of the decreasing enzyme levels over time.

Results

When culture fractions (washed cells or supernatant) were assayed, the proteolytic activity was almost exclusively found in the supernatant (97%) indicating that activity was due to extracellular enzyme or enzymes. The pH optimum for catalytic activity was shown to be pH 9.5 for the two strains (A1 and D5) tested (Fig. 1).

For both strains A1 and D5, enzyme production (measured in terms of both units ml^{-1} and specific activity, units mg dry weight cells) was lowest on TYE plus glucose and highest on either BHI or TYE plus casein (Table 2).

Results (not shown) using gel permeation chromatography calibrated using a range of molecular weight markers showed that activity was probably due to one enzyme with a molecular weight of 44,000 Da. The activity was relatively heat stable at 80°C (half-life of 0.6h at pH 9.5).

Table 2 : Production of trypsin-like protease on various media

Media	Strain A1		Strain D5	
	U ml^{-1}	U mg dry wt^{-1}	U ml^{-1}	U mg dry wt^{-1}
TYE	13.08	5.94	20.58	7.48
TYE + glucose (0.2% w/v)	1.57	2.75	3.28	3.90
TYE + casein (0.2% w/v)	62.40	107.33	47.88	57.00
BHI	87.6	25.03	561.6	244.20

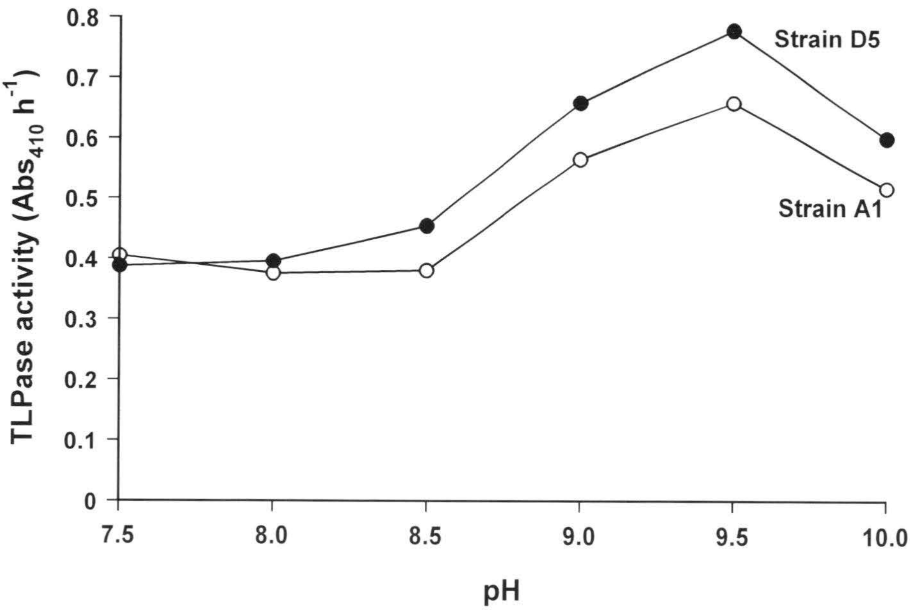


Fig. 1 : pH optima for TLPase activity.

Results using inhibitors and metal ions (Table 3) showed that the enzyme was strongly inhibited by leupeptin but did not require reducing conditions for activity. Magnesium, EDTA and 1,10-phenanthroline were shown to be stimulatory at the concentrations used whereas leupeptin, antipain and Zn^{++} were inhibitory.

Table 3. Effect of enzyme inhibitors and activators on trypsin-like protease

Inhibitor/activator	Final con. (mM)	Residual activity (%)
Control	-	100
EDTA	10	110
1,10-phenanthroline	1	115
$MgCl_2$	5	125
$ZnCl_2$	5	90
Cysteine	5	100
Antipain	5	65
Chymostatin	5	105
Leupeptin	5	10

Discussion

Results presented in this study show that *Stomatococcus mucilaginus* possesses extracellular trypsin-like protease activity. The enzyme (or enzymes) responsible for this activity has a molecular weight close to 44,000 Da. The trypsin-like protease is relatively heat stable, and its pH optima for catalytic activity is pH 9.5. The activity at pH 7.5 is approximately 50% of the maximum activity at pH 9.5. This suggests that at a pH nearer to that encountered on the surface of the tongue, the enzyme could be both active and stable.

In contrast to the cell-bound or vesicle associated (4) trypsin-like protease of *Porphyromonas gingivalis* (5), results using inhibitors and metal ions suggest that the *Stomatococcus* enzyme is not thiol dependent and does not require reducing conditions for activity. The slight stimulation by magnesium ions suggest a possible requirement for this metal ion although EDTA was found to be stimulatory rather than inhibitory.

Experiments comparing protease production on TYE and BI-H show that glucose represses protease production whilst casein induces the expression of enzyme. This suggests that in the protein rich environment of the tongue surface, providing there was an absence of easily available carbon-energy sources such as glucose, the enzyme would be fully expressed. Since the enzyme is both soluble and extracellular it would diffuse within the acellular fraction of the tongue biofilm and attack insoluble as well as soluble protein substrates. This suggests that *Stomatococcus* may play an important role in the hydrolysis of proteinaceous substrates on the tongue and may help supply nutrients to the consortium of organisms present within the tongue biofilm. This may include species which are capable of generating malodour.

References

1. Holt J.G., Krieg N.R., Sneath P.H.A., Staley J.T. and Williams S.T. (1994) Bergy's Manual of Determinative Bacteriology, 9th Edition. Williams & Wilkins, Baltimore. Group 17. Gram-positive cocci. pp. 527-558.
2. Hartley G., El-Maaytah M.A. and Greenman J. (1995) Tongue microflora of subjects with low and high malodour levels. *J Dent Res* 74: 587 (Abstract).
3. Holland K. T., Greenman J. and Cunliffe W. J. (1979) Growth of Propionibacteria on synthetic medium: Growth yields and exoenzyme production. *J Appl Bact* 47:383-394.
4. Minhas T. and Greenman J. (1989) Production of cell-bound and vesicle-associated trypsin-like protease, alkaline phosphatase and N-acetyl-β-glucosaminidase by *Bacteroides gingivalis* strain W50. *J Gen Microbiol* 135:557-564.
5. Yoshimura F., Nishikata M. Suzuki T. Hoover Cl. and Newbrun E. (1984) Characterisation of a trypsin-like enzyme from the bacterium *Bacteroides gingivalis* isolated from human dental plaque. *Archs oral Biol* 29:599-564.

Clinical Assessment and Detection of Breath Odour

Relationship between Volatile Sulphur Compounds and Oral Conditions in the General Japanese Population

*H. Miyazaki, C. Fujita, I. Soh and T. Takehara**

The purpose of this survey was to compensate for the lack of information on the relationship between saliva and oral malodour from the epidemiological point of view, and to obtain more details about periodontal factors. Oral malodour was measured using a portable sulphide monitor in 201 persons aged between 35 and 60 years. In addition, stimulated salivary flow rate and hemoglobin concentration in saliva were measured, and periodontal conditions including bleeding on probing, pocket probing depths and loss of attachment were assessed. Before clinical examination, subjects were interviewed about their medical history and self-diagnosis of bad breath. No correlation was found between actual volatile sulphur compounds (VSC) and self diagnosis. There were no significant differences observed in the VSC values between males and females, and among age groups. No correlation was found between salivary flow rate and VSC.

There was a significant correlation observed between tongue coating and VSC. Periodontal parameters such as hemoglobin concentration in saliva and mean pocket depths could not reflect the level of VSC values in mouth air. However, significant correlations were found between the percentage of sites with periodontal pocket depth of 6 mm or more and VSC, and between the percentage of sites with attachment loss of 6 mm or more and VSC. When both factors of tongue coating and pocket depth were combined, the results suggest that tongue coating might contribute to produce VSC much more than periodontal diseases in this population.

Introduction

The reliability of a portable sulphide monitor which can detect hydrogen sulphide and methyl mercaptan had been tested in several clinical experiments by Rosenberg and co-workers (1, 2). Significant correlations were observed between sulphide concentrations obtained with the monitor and organoleptic ratings by trained examiners. The presently available

---

* H. Miyazaki, C. Fujita, I. Soh and T. Takehara, Department of Preventive Dentistry, Kyushu Dental College, Kokurakita-ku, Kitakyushu 803, Japan.

sulphide monitor is considered to appropriate for assessing oral malodour in epidemiological field surveys despite several limitations.

Oral malodour has been a subject of considerable public interest in many countries, especially in industrialized countries. For example, in a national survey in Japan (3) carried out in 1993, 58% of 33,507 selected persons had some oral problem. Of all the problems, 24% of people aged 30 years and older had complaints relating to oral malodour, and it was the 3rd most frequently observed problem. At the 1st International Workshop on Oral Malodour in 1993, Brenda Schwartz described a national telephone survey in the United States indicating that about 60% of women and 50% of men used cosmetic breath freshening products (personal communication).

Our previous survey in Japan showed that up to 23% of general people showed values of volatile sulphur compounds (VSC) above the socially acceptable limit of 75 ppb at some period during the day. On the other hand, there was no relationship between VSC and self-diagnosis of oral malodour (3). This result combined with the data from a national survey and from persons having actual oral malodour indicates there are many people who have oral malodour and do not know it, as well as many people having it and knowing about it. It might also suggest that quite a few people have problems associated with imaginary bad breath ("halitophobia"). The difficulty in identifying one's own oral malodour may cause confusion. Even clinically healthy people might be affected by the social problems of both "halitophobia" and actual oral malodour.

From our previous survey, periodontal conditions were more severe in men than women in Japan (4). However, no significant difference was shown in VSC between genders (3). This might suggest that for people in the general population with relatively low levels of VSC, oral malodour is not caused by periodontal infections alone but by a combination of several factors, including periodontal conditions, tongue coating, qualitative salivary characteristics and salivary flow rate. It might also be the case that whether periodontitis is currently active is more significant than the mere presence of deeper periodontal pockets, or whether the disease is generalized.

In order to compensate for the lack of information on the relationship between saliva and oral malodour from the epidemiological point of view, the present survey was carried out in 1995. Another purpose was to get more details about periodontal factors. Such epidemiological data in a general population might provide the frame of reference for planning oral health programs from the point of health promotion in community.

Materials and Methods

As a part of annual medical check-ups, in 1995, an oral health survey including oral malodour assessment was carried out. Data from 201 persons (126 males and 75 females) aged between 35 and 60 years working at 4 city halls in Fukuoka Prefecture, Japan were collected. Table 1 shows the distribution of subjects by age group, and gender. The mean age of males and females was 47 years and 48 years, respectively, with no significant difference between genders. All subjects were local government employees, but they were from a wide range of occupational backgrounds such as office workers, teachers, firemen, garbage collectors, etc.

Table 1 : Number of subjects by age group and gender

	Age Group (years)			
	35-44	45-54	55-60	Total
Males	47	61	18	126
Females	24	36	15	75
Total	71	97	33	201

Before the examinations, subjects were interviewed about self diagnosis of oral malodour, smoking habits, medical history including drugs and when they had their last oral activity.

In the oral malodour survey, VSC of each subject were measured with the Halimeter, Model RH I7A (Interscan Corp., Chatsworth, CA, USA). A disposable 60 mm glass tube connected to a plastic tube was attached to the air inlet of the monitor. Each subject was instructed to bring his slightly-opened mouth over the tube so that the tube extended about 4 cm into the oral cavity, and to breathe through his/her nose during measurement. The sample of mouth air passed through a sensor, which registered the level of sulphides. The maximal level attained was determined in parts per billion (ppb) sulphide equivalents by direct readings from the digital display of the monitor. Sensor reliability and reproducibility of the monitor were regularly checked with standard gases of hydrogen sulphide and methyl mercaptan using the Permical Permiator (GL Sciences, Japan).

As a part of periodontal assessments, hemoglobin concentration in saliva was tested using the Salivaster (Showa Yakuhin, Japan). Afterwards stimulated saliva was collected by chewing a paraffin pellet for 5 minutes and the volume over time (ml/min) was calculated as a salivary flow rate.

A trained dentist assessed the subjects' periodontal conditions including

bleeding on probing, pocket probing depths and loss of attachment in mm at six sites of all teeth except for third molars using a medical halogen head light, dental mirrors and Sensor probes in a room of each city hall.

Subjects were also assessed for their tongue coating status according to the area involved. Score 0: none visible; 1: less than one-third of tongue dorsum surface was covered; 2: less than two-thirds; 3: more than two-thirds.

Statistical analysis of the data was based on Mann-Whitney U-test or Post-hoc analysis for the mean value of VSC. Spearman correlation analysis was used to determine the relationship between the VSC and each variable.

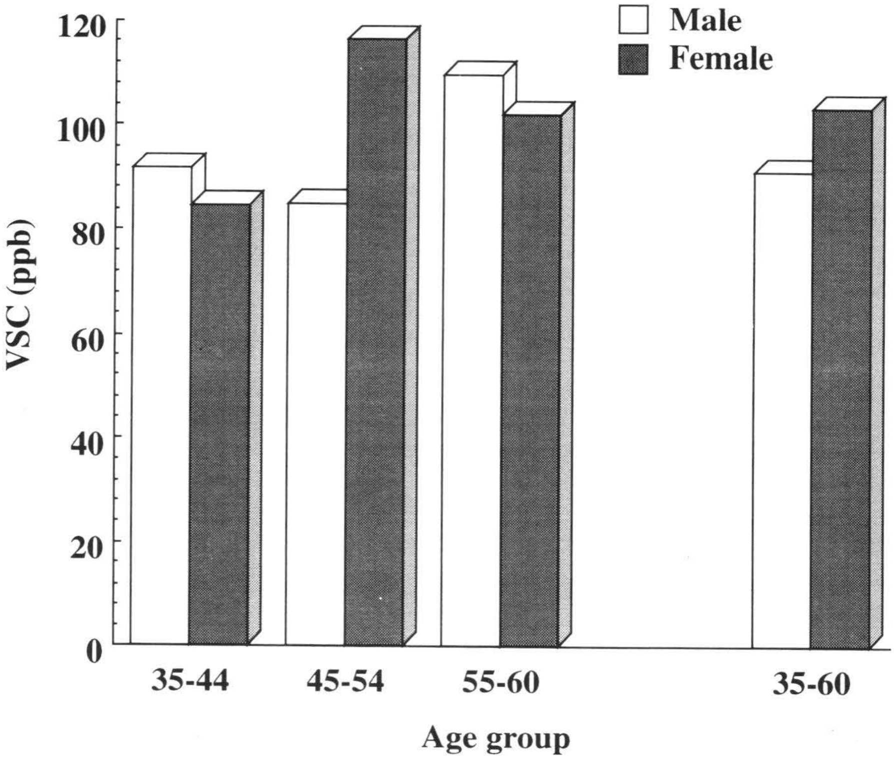


Fig. 1 : Mean VSC by age group and gender

Results

Fig. 1 shows the mean VSC values by age group and gender. Mean VSC was 95.6 ppb among all subjects examined. Mean VSC values showed no

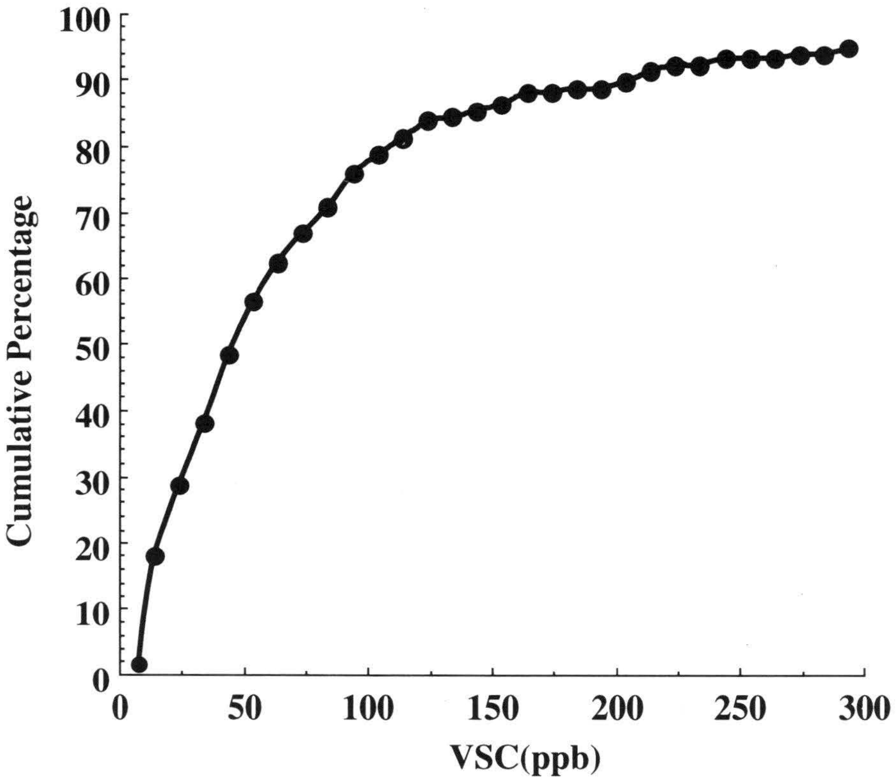


Fig. 2 : Distribution of VSC and cumulative percentage in 201 subjects.

significant differences both between age groups and between genders. Therefore, in the following analyses, the VSC values were used without consideration of gender and age factors.

Fig. 2 illustrates graphically the distribution of the VSC value of all subjects. 50 percentile of persons showed VSC of about 50 ppb. 76% of persons had less than mean VSC of 96 ppb in this population. And, 32% of subjects had VSC values above the suggested socially acceptable limit of 75 ppb.

Fig. 3 shows the distribution of subjects by stimulated salivary flow rate in ml/min. The largest group (55 persons or 27%) had a salivary flow rate of 1.0-1.5 ml/min. followed by the 1.5-2 .0 and the 2.0-2.5 ml/min. groups.

When tentatively divided into 3 groups, such as salivary flow rate of less than 1 ml/min., between 1 and 3 ml/min. and more than 3 ml/min., as shown in figure 4, mean VSC value of each group was 97 ppb, 98 ppb and 83 ppb, respectively. VSC level was 14% less in the group of more than 3 ml/min. than the other groups. However, the difference was not significant.

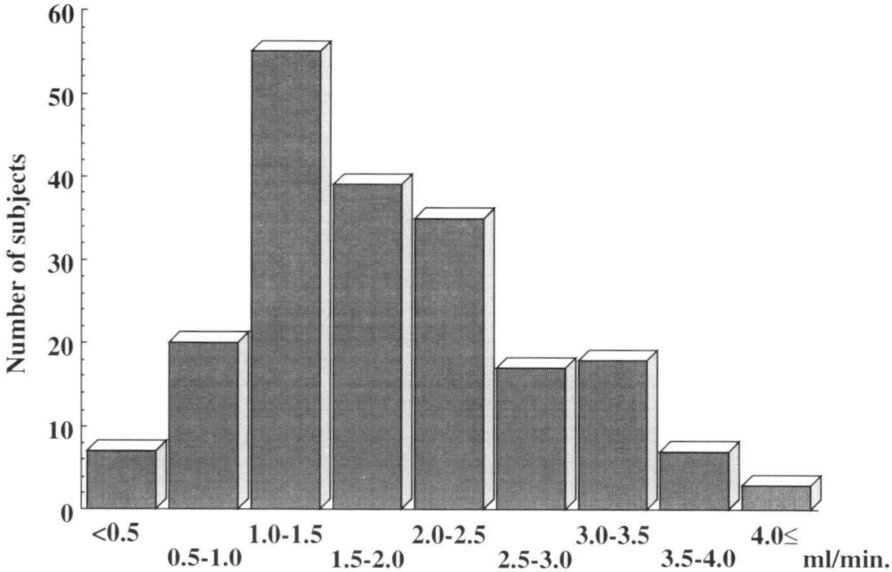


Fig. 3 : Distribution of subjects according to salivary flow rate.

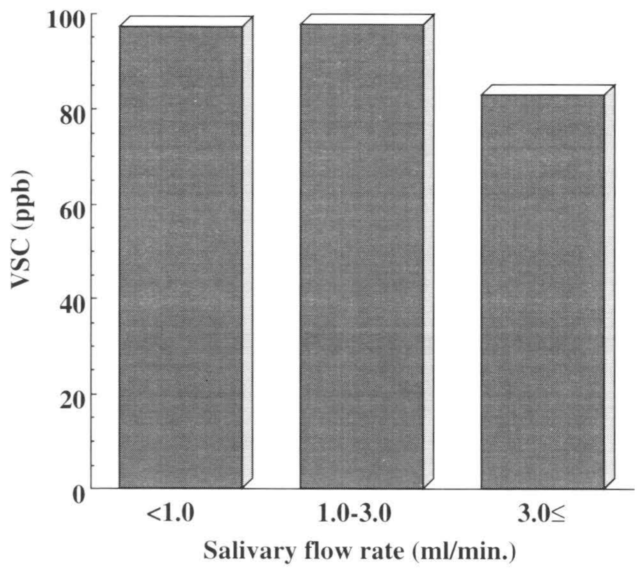


Fig. 4 : Mean VSC of persons according to salivary flow rate.

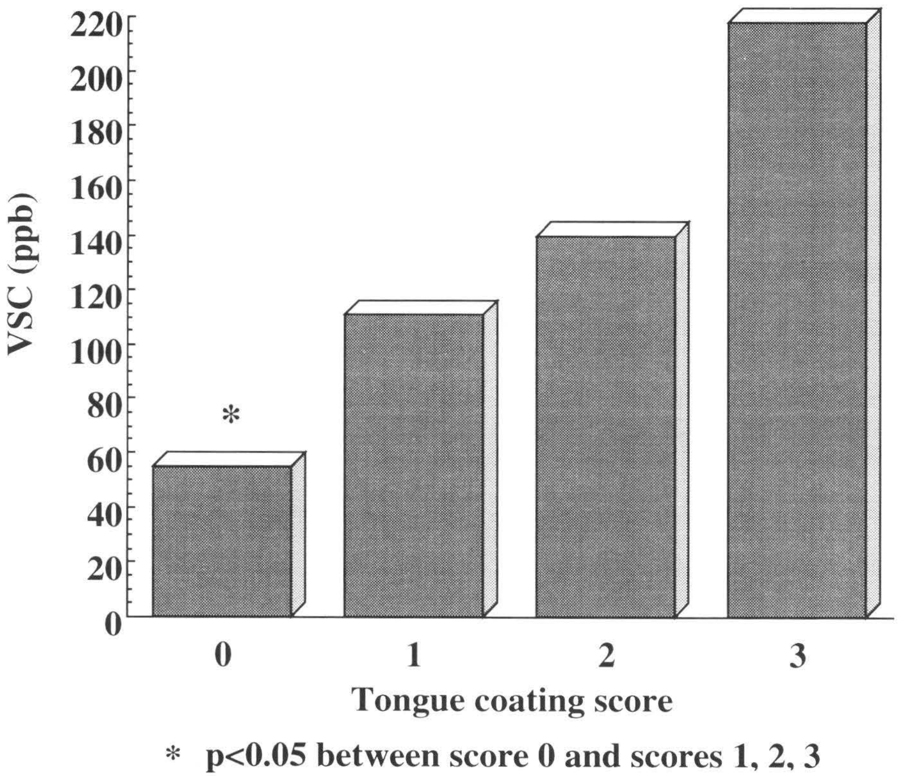


Fig. 5 : Mean VSC of persons according to tongue coating.

Fig. 5 shows mean VSC values by persons with different score of tongue coating. Eighty-four % of persons had tongue coating with less than one third of tongue dorsum, and only 3% had tongue dorsum of more than two-thirds with coating. VSC level tended to be higher with tongue surface area with coating. Especially, VSC was significantly lower with tongue coating score of 0 than for 1, 2 or 3.

Fig. 6 shows the mean VSC of persons with different levels of hemoglobin concentration in saliva. Mean VSC tended to increase with higher hemoglobin concentration, but significant differences were not found among 3 groups.

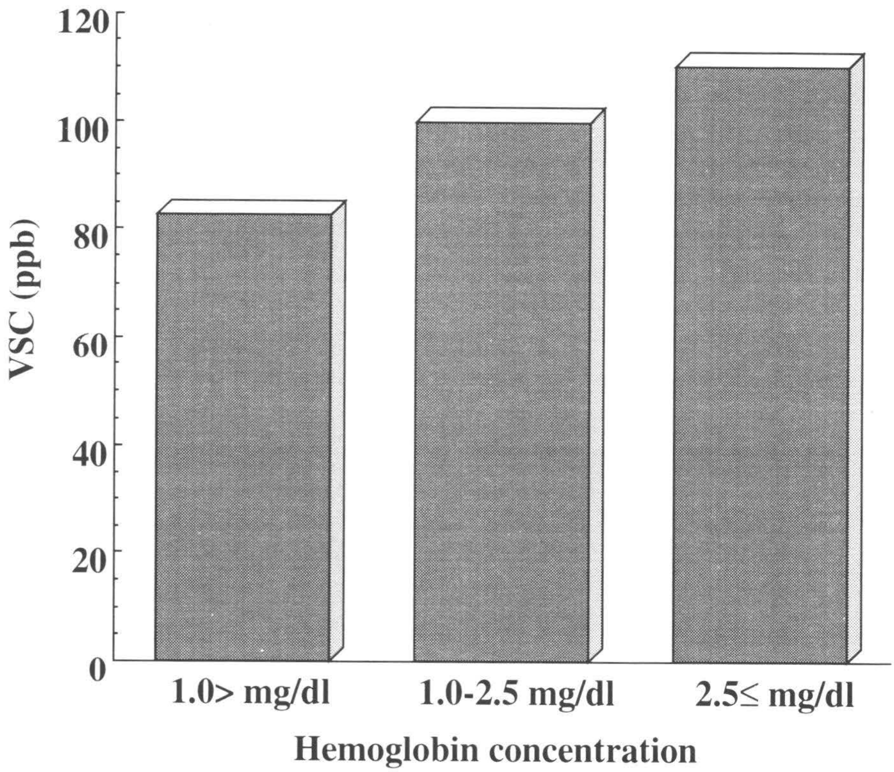


Fig. 6 : Mean VSC of persons by hemoglobin concentration in saliva.

Figs. 7 and 8 show the mean VSC by group based on periodontal pocket depth and based on loss of attachment. Both in pocket depths and loss of attachment, VSC values showed no significant difference among 3 groups. When periodontal patients were defined according to the maximum value of pocket depth or attachment loss, the Spearman correlation coefficient comparing VSC and maximum pocket depth was 0.155 ($p < 0.05$), and 0.182 ($p > 0.05$) comparing VSC and maximum attachment loss.

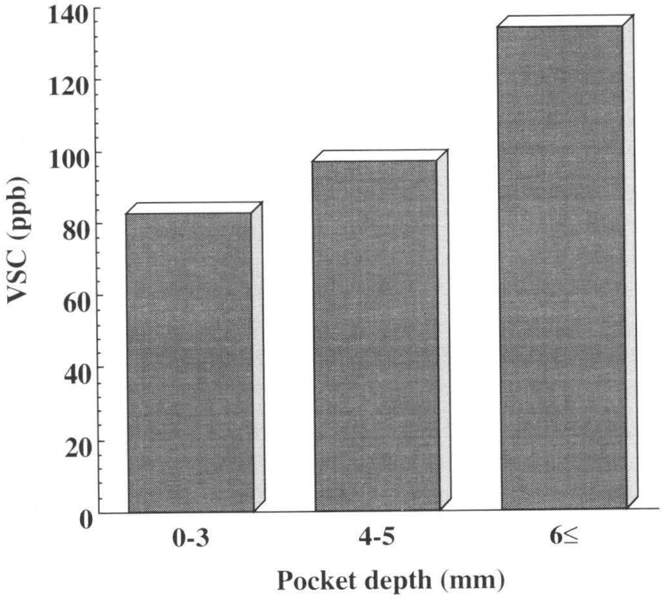


Fig. 7 : Mean VSC in subject groups with at least one site of pocket depth categorized into 3 groups.

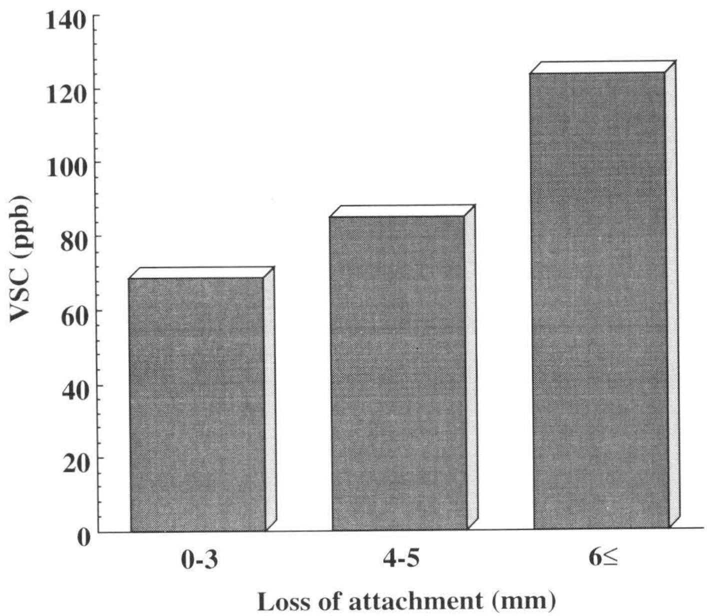


Fig. 8 : Mean VSC in subject groups with at least one site of attachment loss categorized into 3 groups.

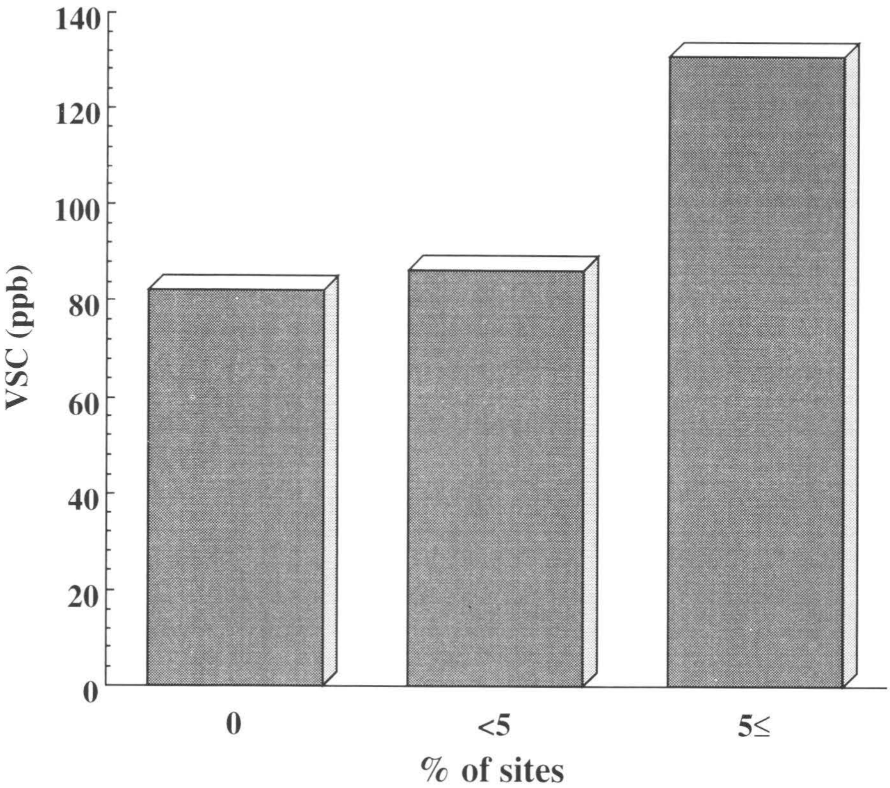


Fig. 9 : Mean VSC by percentage of sites with pocket depths of 4mm or more.

Figures 9 and 10 show the mean VSC by percentage of sites with pocket depths of 4 mm or more, and with pocket depths of 6 mm or more, respectively. As shown in Figure 9, mean VSC was similar between persons having no pockets of 4 mm or less and persons having less than 5% of sites with pocket depths of 4 mm or more. On the other hand, mean VSC showed 1.5 times higher in the group of persons having more than 5% of sites with pocket depths of 4 mm or more than in the other groups. In Figure 10, persons having more than 5% of sites with pocket depths of 6 mm or more showed 208 ppb of VSC level, which was 1. 7 times higher than that having less than 5% of sites and 2.3 times higher than that having no pockets of 6 mm or more. When the Spearman correlation was calculated, the correlation coefficient between VSC and percentage of sites with pocket depths of 6 mm or more was 0.386, and it was significant.

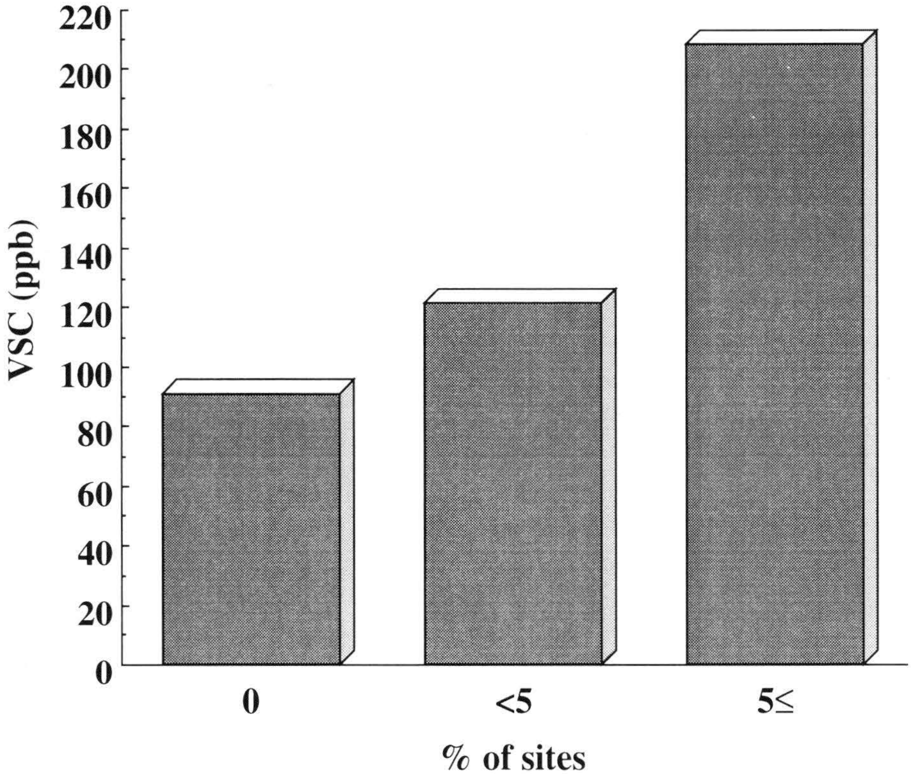


Fig. 10 : Mean VSC by percentage of sites with pocket depths of 6 mm or more.

Discussion

In an ordinary population group there is little influence of salivary flow on oral malodour. We can find oral malodour patients in clinics, who have no cause for their oral malodour except for a salivary problem, especially xerostomia patients having Sjögren syndrome (5, 6) or after radiotherapy in the maxillo-facial area (7, 8). However, such people are scarcely included in population group. At least from the results of distribution of subjects by salivary flow rate, persons having too little or too much saliva secretion are considered to be only a minority in the general population. For the present survey, people using drugs, many of which might reduce salivary flow (9, 10, 11), were omitted.

The association between oral malodour and periodontitis has been regularly reported (12, 13, 14, 3). On the other hand, many studies also indicated that tongue coating played more important role than periodontitis on VSC production (15, 16, 13, 14, 17, 3). In the present survey, periodontal

conditions were worse in men than women on the basis of mean number of sites of bleeding on probing, pocket depths and attachment loss. However, no significant difference was shown in VSC between genders, similar to a previous report (3). When analyzing the correlation between periodontal conditions and VSC, both negative and positive results can occur according to which periodontal indicator is selected. For example hemoglobin concentration in saliva which might reflect bleeding from gingival tissue, deep pockets or attachment loss were not related to VSC values in mouth air. VSC from periodontal pockets may not be detected in whole mouth air (17). On the other hand, a positive but weak correlation between them seems to be found when the number of sites with periodontitis is used as a quantitative assessment. Therefore, it is doubtful that VSC measurement in mouth air should be considered as a diagnostic marker for periodontal infections.

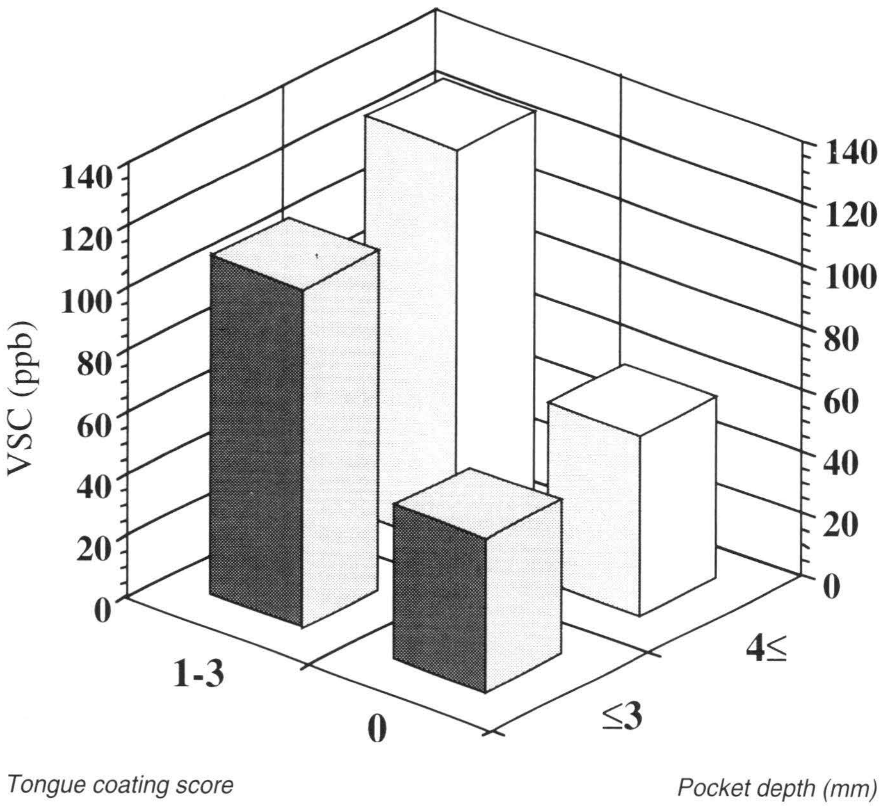


Fig. 11 : Mean VSC values by group combined tongue coating score (0 or 1-3) and pocket depth (0-3 mm or 4 mm or more).

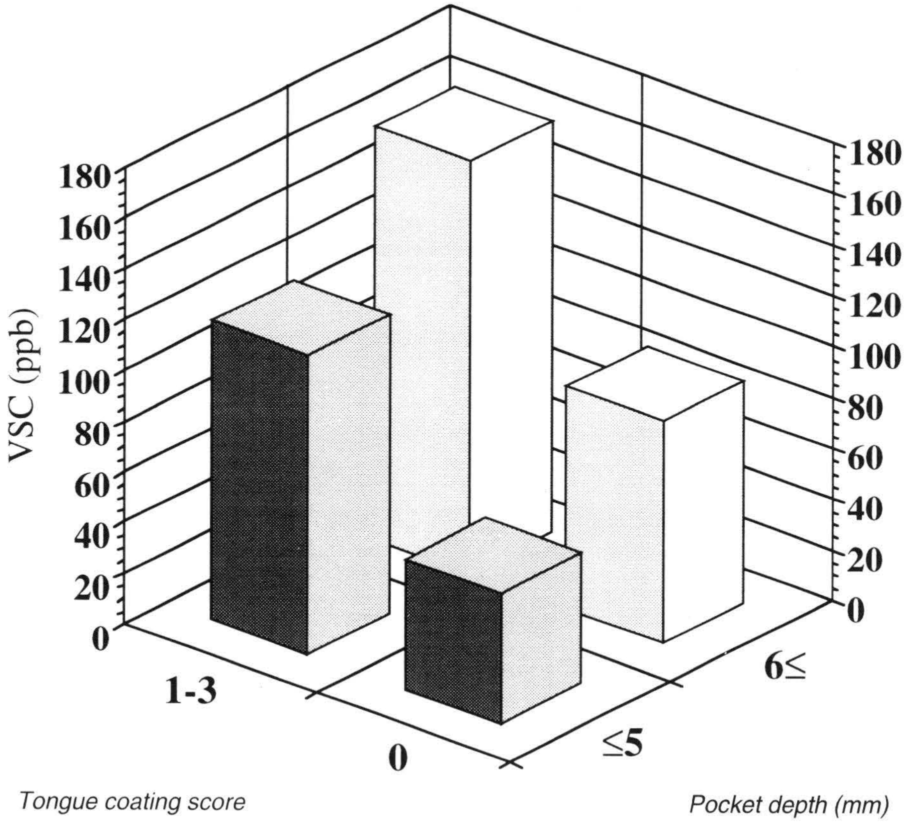


Fig. 12 : Mean VSC values by group combined tongue coating score (0 or 1-3) and pocket depths (0-5 mm or 6 mm or more)

In order to clarify which major causes, such as tongue coating and periodontitis contribute to VSC productions, both factors were combined to establish 4 groups. Fig. 11 shows the mean VSC by group, that is, no tongue coating and no pockets more than 4 mm, no tongue coating and pockets of 4 mm or more, tongue coating and no pockets more than 4 mm, tongue coating and pockets of 4 mm or more. In Fig. 12, pocket depth of 6 mm is used as a threshold of periodontitis. In both figures, persons with both tongue coating and periodontal pockets had highest VSC values. Persons with tongue coating but no pockets had higher VSC scores than those with no tongue coating but periodontal pockets. These results suggest that tongue coating might contribute more to the production of VSC than periodontitis in this population.

In conclusion, 32% of subjects showed VSC values above the suggested socially acceptable limit. No correlation was found between actual VSC and self-diagnosis of bad breath. There were no significant differences observed in the VSC values between males and females, and among age groups. There was a significant correlation observed between tongue coating and VSC. Significant correlations were found between the percentage of sites with periodontal pocket depth of 6 mm or more and VSC, and between the percentage of sites with attachment loss of 6 mm or more and VSC. No correlation was found between salivary flow rate and VSC.

References

1. Rosenberg M., Kulkarni G.V., Bosy A. and McCulloch C.A.G. (1991) Reproducibility and sensitivity of oral malodour measurements with a portable sulphide monitor. *J Dent Res* 70:1436-1440.
2. Rosenberg M., Septon I., Eli I., Bar-Ness R., Gelernter, I., Brenner, S. and Gabbay, J. (1991) Halitosis measurement by an industrial sulphide monitor. *J Periodontol* 62:487-489.
3. Miyazaki H., Sakao S., Katoh Y. and Takehara T. (1995) Correlation between volatile sulphur compounds and certain oral health measurements in the general population. *J Periodontol* 66: 679-684.
4. Miyazaki H., Hanada N., Andoh M.I. *et al.* (1989) Periodontal disease prevalence in different age groups in Japan as assessed according to the CPITN. *Community Dent Oral Epidemiol* 17:71-74.
5. Gumpel J. (1982) Sjögren's syndrome. *Br Med J* 285:1598.
6. Edgar W.M. (1992) Saliva: its secretion, composition and functions. *Br Dent J* 172: 305-312.
7. Fox P., Weiffenbach J., van der Ven P., Baum B. and Sonies B. (1985) Xerostomia: evaluation of a symptom of increasing significance. *J Am Dent Ass* 110: 519-525.
8. Schubert M. and Izutsu K.T. (1987) Iatrogenic causes of salivary gland dysfunction. *J Dent Res* 66:680-688.
9. Christensen C.M., Navazesh M. and Brightman V.J. (1984) Effects of pharmacologic reductions in salivary flow on suprathreshold judgments of taste stimuli. *Archs oral Biol* 29: 17-23.
10. Thorselius I., Emilson C. and Osterberg T. (1988) Salivary conditions and drug consumption in older age groups of elderly Swedish individuals. *Gerodontics* 4: 66-70.
11. Sreebny L.M. and Valdini A. (1989) Xerostomia. Part II: Relationship

to nonoral symptoms, drugs and diseases. *Oral Surg Oral Med Oral Pathol* 68: 419-427.

12. Kostelc J.G., Preti G., Zelson P.R., Brauner L. and Baehni P. (1984) Oral odors in early experimental gingivitis. *J Periodont Res* 19:303-312.
13. Yaegaki K. and Sanada K. (1992) Biochemical and clinical factors influencing oral malodor in periodontal patients. *J Periodontol* 63:783-789.
14. Yaegaki K. and Sanada K. (1992) Volatile sulfur compounds in mouth air from clinically healthy subjects and patients with periodontal disease. *J Periodont Res* 27:233-238.
15. Tonzetich J. and Ng S.K. (1976) Reduction of malodor by oral cleansing procedures. *Oral Surg Oral Med Oral Pathol* 42:172-181.
16. Kleinberg I. and Westbay G. (1990) Oral malodor. *Crit Rev Oral Biol Med* 1:247-260.
17. Bosy A., Kulkarni G.V., Rosenberg M. and McCulloch C.A.G. (1994) Relationship of oral malodor to periodontitis: evidence of independence in discrete subpopulations. *J Periodontol* 65: 37-46.

Oral Malodour in the Elderly

*W.J. Loesche, N. Grossman, L. Dominguez and M. A. Schork**

We are conducting a longitudinal investigation of the relationship between oral health and medical health in a convenience sampling of individuals over 60 years of age, who are living in southeast Michigan. Among the issues being studied is the prevalence of complaints of oral malodour and reasons for such among these individuals. The prevalence of malodour as elicited by a positive response to the question "Have you been told that you have bad breath?" was 24% (66 positive responses from 270 respondents), whereas 31% (83/270) thought that they had "bad breath". Both prevalences suggest that concerns over malodour are high in the elderly.

There was a tendency for individuals who were current smokers to have been told that they had "bad breath". The use of full dentures and being edentulous could not be associated with complaints. However, the wearing of partial dentures in the mandibular arch was significantly associated with subjects being told that they had "bad breath". There were no differences in the number of decayed teeth, in gingivitis (papillary bleeding), in plaque scores, or in resting or stimulated salivary flow between individuals with and without complaints. There was no association of complaints with periodontitis as measured by probing depths or attachment loss. There was no association of complaints with oral hygiene practices such as the number of times the teeth were brushed or flossed, or visits to the hygienist for dental cleanings.

These findings suggest that there are few tooth, or hygiene, or saliva based parameters associated with complaints of oral malodour. The few that were found in the individuals who were told that they have "bad breath", i.e., wearing of partial dentures by individuals with > 14 teeth do not contradict this conclusion. Thus while oral malodour seems to be a common complaint among the elderly, there is little data to suspect the teeth or dentures as being important contributors to the problem.

---

* W.J. Loesche, N. Grossman, L. Dominguez and M.A. Schork, Oral Health Sciences, School of Dentistry, University of Michigan, Ann Arbor, Michigan 48109-1078, USA.

Introduction

Complaints of oral malodour are thought to be common among individuals who seek dental care (1), but minimal information concerning its prevalence in the general population is available. There are no epidemiological surveys which address this concern with the exception of recent Japanese data involving 2,601 individuals, which indicated that as many as 25% of the subjects had oral malodour as measured by volatile sulphur compounds higher than 75ppb in expired air (2). If these Japanese data reflect the prevalence of oral malodour in other populations, then oral malodour would represent a major oral health concern of the public.

We sought to obtain some information on the prevalence of complaints of oral malodour among older US subjects, who were being studied as part of an epidemiological investigation of the interactions between oral/dental health and medical health (3). The subjects were asked whether they thought that they had "bad breath", or were told that they had "bad breath". This approach was based on the observation that many individuals with oral malodour do not recognize that they have malodour unless they are "told" of it, whereas individuals who "think" that they have malodour, often have no objective evidence for it (4).

Initial studies of individuals with oral malodour tended to relate the malodour to the presence of periodontal disease (5, 6). More recent studies, however, indicate that the tongue is the major source of the malodour (7-10), and in some cases the malodour could be related to the amount of coating on the tongue (6, 10). This coating would contain large numbers of bacteria that are capable of producing the malodorous compounds (see chapter 9). In the present investigation we determine whether the complaint of malodour could be related to the many oral, dental, salivary and medical parameters that were being collected as part of the larger study (3).

Materials and Methods

Subjects

The subjects were 60 years or older and resided in southern Michigan. The choice of subjects was determined in part by the intention to have groups of older individuals with a variety of medical backgrounds, and lifestyles. About 70% of the subjects were recruited from the dental outpatient clinic, the long

term care facility and acute care clinic of the Ann Arbor Veterans Administration Hospital (VA subjects), while the remainder were drawn from a private retirement home and lists of volunteers maintained by the University of Michigan Geriatric Center. As such, convenience groups were selected, and the results cannot be generalized to all individuals of similar age.

The majority of the subjects (65%) were living independently in the community, whereas the remainder were dependent living, either in a nursing home (14%), or had recently been admitted to an acute care ward (21 %) with a medical condition which had created a swallowing problem. These latter patients were a unique group that had been recruited because of the study's interest in the relationship between oral problems and the development of pneumonia in the elderly (3). Due to this interest we had extensive data on salivary and swallowing parameters and the use of xerogenic medications (11). The subjects were mostly white (77%) and male (78%). Detailed demographic characteristics and oral findings of the subjects are presented elsewhere (3).

Questionnaire

All participants were interviewed by trained examiners who used a structured questionnaire to elicit information about demographic characteristics, medical and dental history and use of medications. The interview lasted from one to one and a half hours and detailed information regarding the subject's eating habits, food and liquid preferences, oral hygiene habits, smoking and drinking tendencies, complaints of oral malodour and xerostomia, swallowing complaints, medical history and usage of prescription medications was obtained.

Dental/Periodontal Examination

One dentist/hygienist team examined the VA subjects and a second team examined the non-VA subjects. The number of teeth that were present and the number of decayed/missing/filled teeth and surfaces (DMFR, DMFS) were diagnosed on the basis of criteria described in the national survey on the oral health of US adults (12). The presence of any fixed or removable prostheses, dentures or implants was recorded. Gingivitis was assessed by a papillary bleeding score (PBS) (13) about each interproximal site in the dentition. An average PBS was obtained by adding the scores for each interproximal site and

dividing the total by the number of sites examined. The plaque score was obtained with the use of the Plaque Index (PI) (14). The probing depths about 6 surfaces on each tooth were obtained with a constant pressure probe (Interprobe, Bausch and Lomb, Rochester, NY). The number of teeth varied greatly among the subjects. In order to account for this variability the probing depths and attachment losses per patient were normalized as % of sites < 3.5mm, % of sites 4 to 6.5mm, and % of sites > 6.5mm.

Saliva

Whole saliva was stimulated by swabbing the tongue with a 2% citric acid solution three times at 30-second intervals (11). If the subject was unable to provide about 1 ml of saliva after 5 minutes, he or she was given a default value of < 0.2 ml saliva per minute. This method of stimulating saliva yields lower values than that obtained by chewing paraffin wax, but was chosen because of the high number of missing teeth and dentures among our subjects, which gave an uneven chewing performance (15). Also there was a concern that some subjects might aspirate the wax. The minor salivary gland output was measured as the degree of wetness present in standardized cellulose strips (15), that were placed for 30 seconds on the hard palate, the buccal mucosa, and inside the lower lip. The findings were reported as μL/60 seconds for each location, and the sum of the three locations was reported as the minor salivary gland output.

After the stimulated saliva was collected and weighed, it was frozen in liquid nitrogen until cultured. Batches of 10 or more salivas were thawed, serially diluted in reduced transport fluid, and various dilutions were inoculated, using a spiral plater, on enriched trypticase soy agar (ETSA) medium. Duplicate ETSA plates were incubated at 35°C, either in air, or in an anaerobic chamber under an atmosphere of 85% nitrogen, 10% carbon dioxide and 5% hydrogen. After 7 days incubation the number of colony forming units per ml of saliva on both the aerobically and the anaerobically incubated plates was determined and log transformed (17).

Statistical Analysis

We were interested in how the subjects' responses to the question "Do you think that you have "bad breath"?", or "Have you been told that you have "bad breath"?", would be reflected in the level of their dental/periodontal morbidity.

We found that the answers to these questions resulted in 4 response groups: subjects who had neither been told or thought that they had "bad breath", i.e., "no complaint group"; subjects who thought that they had "bad breath", i.e., "think group"; subjects who were told that they had "bad breath", i.e., "told group"; and subjects who had been told and who also thought that they had "bad breath", i.e., "told and think group". Pearson's Chi-square test was used to make overall comparisons of the categorical data between the groups defined by their response to the above questions. Fisher's exact test was used to make pairwise comparisons between proportions. Overall comparisons between groups of continuous variables were made with the use of a one-way analysis of variance. The various statistical analysis were performed using SAS programs.

Table 1: Prevalence of Complaints of Oral Malodour

		% of Individuals	
Complaint	Number	Think	Told
None	155	57	
Think have	49	18	
Told have	34	13	24
Told & Think have	32	12	

Results

Forty three percent of the subjects reported either thinking that they had "bad breath" or were told that they had "bad breath". Thirty percent thought that they had "bad breath" either because they were told of it (13%), or because of other reasons (18%) (Table 1). Twenty five percent had been told that they had "bad breath", but over half of these individuals (13%) did not think that they had "bad breath", i.e., "told group" in Table 2. The complaint of "bad breath" could not be related to the race of the subject, or to the level of education. There was a tendency for males and for independent living subjects to have more complaints, especially among those who were told that they have "bad breath".

Table 2: Perceived Causes of Oral Malodour among Individuals who "Think" they have "Bad Breath"

Perceived Cause	Number of individuals	Think bad breath n = 39-42[a]	Think and told bad breath n = 27-39[a]
Smoking	9	5[b]	10
Foods	21	21	11
Dentures or teeth	31	26	22
Morning breath	48	39	28

[a] number varies because some individuals did not answer all questions related to perceived causes
[b] The percentage of all subjects who think that they had bad breath.

Table 3 : Relationship between Smoking Status and Perceived Presence of Oral Malodour

			Prevalence of complaint (%)				
Smoking status	(n)	(%)	None	Think	Told	Told and think	Significance
Never smoked	73	26	17[a]	6	1	2	
Quit smoking	154	57	33	10	7	7	
Current smoker	46	17	7	2	5	3	
Total	273	100	57	18	13	12	p = 0.01

[a] percent of total subjects

The subjects who thought that they had "bad breath" were asked if they could associate the malodour with any cause. (The individuals who were told that they had "bad breath", but didn't think so, did not respond to these questions). Only 15% associated the malodour with smoking, 32%, with various foods, 48% with dentures, but 67% associated it with morning breath (Table 2). There was no difference in these associations between those subjects who thought that they had "bad breath", and those who were told and thought that they had "bad breath" (Table 2). The relationship between smoking and "bad breath" could be assessed by looking at the patient's current smoking status. Only 17% of the subjects were current smokers and these subjects tended to be in the "told" and "told and think" groups (Table 3).

Table 4: Relationship between Reported Hygiene Practices and Complaints of Oral Malodour

Hygiene practice (number of subjects who responded)		Prevalance of complaint (%) None	Think	Told	Told and Think	Significance
Brush teeth ≥ 1/day	(194)	96	96	96	**84**[a]	0.075
Clean denture ≥ 1/day	(138)	87	89	90	87	0.33
Visit hygienist ≥ 1/yr	(195)	75	77	67	84	0.75
Never floss	(195)	28	29	48	28	0.3
Never clean tongue	(245)	34	31	41	36	0.3

[a] value in bold significantly different from other values in row. Fisher's exact test.

Poor oral hygiene would be expected to contribute to oral malodour. Eighty four percent of the individuals in the "told and think" group brushed their teeth at least once per day, compared to 96% in the other groups (Table 4). This difference was statistically significant, but the absolute differences between this group and the others was so liminal that this would not seem to explain the occurrence of malodour in this group. Likewise, other hygiene parameters, such as the frequency at which dentures were cleaned, or visits to the hygienist did not distinguish between the no complaint group and the various complaint groups (Table 4). The number of individuals who never flossed or never cleaned their tongues was comparable in the no complaint group and the various complaint groups (Table 4).

Periodontitis might account for the prevalence of malodour in those individuals with complaints. However, there was no evidence of more gingivitis or periodontitis in the individuals with a complaint of malodour than in those without a complaint (Table 5). The plaque scores, the PBS and the plaque BANA scores were comparable in all groups. There was no overall difference between the groups in terms of the number of teeth with pockets 4 to 6.5 mm or with pockets > 6.5. However, in the pairwise comparisons the individuals in the "told and think" group had significantly more pockets in the 4 to 6.5 mm range than did the individuals who had no complaints (Table 5). When the probing measurements were normalized as a percentage of tooth sites with various levels of morbidity, there were no differences between the groups for either probing depths or attachment loss. The data for percent of teeth with various levels of attachment loss indicated no differences between the groups (Table 5).

Table 5 : Relationship between Periodontal Parameters and Complaints of Oral Malodour

	Prevalence of complaint				
Periodontal parameter	None	Think	Told	Told and think	Significance
Plaque score	2.4	2.3	2.6	2.4	0.7
Papillary bleeding score	2.2	2.2	2..4	2.1	0.8
Plaque BANA	1.6	1.6	1.5	1.7	0.8
n° of teeth with					
pockets 4 to 6.5 mm	4.9[a]	5.0	5.4	7.0[a]	0.2
pockets ≥ 7 mm	0.5	0.4	0.2	0.5	0.6
% of teeth with attachment loss					
< 3.5 mm	46%	51%	45%	42%	0.7
4 to 6.5 mm	43%	43%	49%	46%	0.7
≥ 7 mm	11%	6%	6%	12%	0.3

[a] *groups are significantly different (ANOVA)*

Table 6 : Relationship between Decay Parameters and Complaints of Oral Malodour

	Complaint				
Decay parameter	None	Think	Told	Told and think	Significance
n° of decayed teeth	1.8	2.1	1.9	2.0	0.97
% decayed surfaces	3.7	2.1	3.4	1.6	0.56
% decayed root surfaces	2.9	1.7	**6.6**[a]	0.9	0.24
% teeth ache	5.4	1.7	7.4	11.5	0.66

[a] *value in bold significantly different from other values in same row*

Dental decay could also contribute to oral malodour. There were no differences between the individuals with a complaint and those without a complaint in regards to the number of decayed teeth, and the percentage of decayed coronal or decayed root surfaces (calculated as the number of decayed surfaces over the total number of coronal or root surfaces at risk to decay) (Table 6). There was a significant increase in the percent of decayed root surfaces in the "told" group compared to the other groups, but this did not seem to be related to a complaint of malodour, as there was no tendency to have higher percent of root surface decay in the other malodour groups (Table 6).

Many of the subjects wore partial and complete dentures. There was a tendency for individuals who wore a maxillary partial denture to have a

complaint of oral malodour (Table 7). Subjects who wore a mandibular partial denture were significantly likely to have been told that they had "bad breath" (Table 7). The wearing of full dentures could not be associated with complaints of oral malodour.

Table 7 : Relationship between Presence of Dentures and Complaints of Oral Malodour

		Prevalence of complaint (%)				
Presence of denture (number of subjects)		None	Think	Told	Told and think	Significance
Maxillary Arch						
None	(109)	59	16	14	12	
Partial	(31)	39	23	16	23	
Full	(101)	58	20	14	8	p = 0.3
Mandibular Arch						
None	(135)	56	19	13	13	
Partial	(42)	50	7	**26**	**17**[a]	
Full	(64)	63	23	9	5	p = 0.03

[a] *value in bold significantly different from other values in row.*

The saliva could affect oral malodour in several ways. If the salivary flow were reduced, then there might be more time for the oral microbes to ferment salivary glycoproteins to malodorous sulphur compounds and volatile fatty acids. There would be reduced elimination of oral bacteria via swallowing, and this would increase the bacterial load on the oral surfaces which could contribute to the production of more malodorous compounds. However a scenario for increased production of malodorous compounds can also be made if the salivary flow was normal or increased. In this case even though there would be increased elimination of oral bacteria, there would be the possibility that the increased flow would bring more nutrients to the flora and that this would increase growth and concurrent production of malodorous compounds.

These possibilities were examined by measuring both the stimulated salivary flow and the number of anaerobic and non-anaerobic bacteria that could be cultivated per ml of saliva. There was a tendency for the stimulated and resting salivary flow to be higher in the individuals who had been "told" that they have "bad breath" (Table 8). Both these increased flows could be associated with a reduced density of the anaerobic and the nonanaerobic flora in the saliva (Table 8). However there was no overall pattern between salivary flow and the complaint of "bad breath", nor between bacterial density of the saliva and "bad breath".

Table 8 : Relationship between Salivary Parameters and Complaints of Oral Malodour

	Complaint				
Salivary parameter	None (155)[a]	Think (49)	Told (34)	Told and think (32)	Significance
Stimulated Saliva (ml/min)	0.65	0.60	**0.78**[b]	0.64	0.16
Minor salivary gland output (µl/min)	2.29	2.4	2.72	2.56	0.28
Log10 anaerobic count/ml	8.07	8.03	7.89[c]	8.27[c]	0.19
Log10 aerobic count/ml	7.81	7.75	7.64	7.86	0.60
Median number of medicines	6.0	7.0	5.5	6.1	0.48
Xerogenic medicines	1.1	**1.7**	1.25	**1.65**	0.008

[a] *number of subjects in each complaint group*
[b] *values within bold are significantly different from other values in row*
[c] *values with same superscript are significantly different*

These older subjects took on a regular basis an average of 5 to 6 medications, some of which are known to reduce salivary flow (12). Individuals in the "think" and "told and think" groups used significantly more xerogenic medications, than the subjects in the "told" or "no complaints" groups (Table 8). The subjects who were told that they had "bad breath" used fewer xerogenic medications which could account for their higher salivary flow and reduced bacterial densities per ml saliva (Table 8).

The high usage of medicines in this group of older individuals raised the possibility that medical conditions could be responsible for the complaints of malodour. However, the medical histories revealed no obvious connection between the complaint of "bad breath" with any cardiovascular, neurological, diabetic or respiratory condition. There was a significant positive association between an allergic history and a complaint of malodour, especially in those individuals in the "think and told" group.

Discussion

The present study recorded a complaint of malodour, and not the presence of malodour, as there was no independent measurement of malodour, either via a sulphide monitor, or by an organoleptic measurement (7-9). Also as the subjects were not randomly selected and represented convenience sampling, the results can not be generalized to all older subjects, or to the public at large. Nevertheless, this survey suggests that the complaint of malodour is very common among older individuals, being second only to a complaint of

dry mouth (11). With 43% of the subjects reporting a complaint, malodour is more common than a complaint of a toothache (Table 6), and of bleeding or sore gums i.e., 15% (data not shown). A similar high prevalence of oral malodour has been reported amongst the Japanese (2). It also confirms a recent survey of US dentists which reported that 41% of the dentists see 6 or more patients a week with chronic "bad breath" (1).

The present findings should dispel some of the myths associated with the causes of oral malodour. For example in the survey of dentists cited above, it was reported that "63% of the dentists indicate that lack of good oral hygiene is the leading cause of "bad breath", and 51% say that the most effective treatment is regular dental cleanings with oral hygiene instruction" (1). There is no evidence amongst our subjects that oral hygiene played a role in the malodour (Tables 4 and 5). The subjects with a complaint of "bad breath" brushed their teeth daily, cleaned their dentures, and visited their hygienist at least once per year, at the same frequency that individuals without a complaint did. The only situation where poor oral hygiene could be contributing to the oral malodour was in those individuals wearing a partial denture (Table 7), if we assume that oral hygiene is more difficult to achieve in the presence of partial dentures. The findings from the Japanese study also could not show a relationship between oral volatile sulphur compounds and tooth brushing habits (2).

The cited survey (1), reported that "21% of the dentists ranked periodontitis as the second most common cause of" bad breath. However among our subjects there was no evidence that periodontitis contributed appreciably to the oral malodour. The plaque scores, gingivitis scores (PBS) and periodontal morbidity of those individuals with a complaint were comparable to those observed in individuals without a complaint (Table 5). At least five other groups have been unable to associate periodontitis with oral malodour (2,7-10). In these studies the tongue, and more specifically the amount of coating on the tongue (6,10,11), has been implicated as the major cause of oral malodour. There is often heavy tongue coatings when there is periodontitis (6). However when the presence of these coatings on the tongue is accounted for, any contribution of the periodontal condition to the oral malodour disappears (10).

When the many measured dental, periodontal and salivary parameters were compared between the four response groups, there were few differences found between subjects who did not have a complaint and those with a complaint. There was a tendency for the subjects in the "think and told" group to brush less frequently (Table 4) and to have more pockets in the 4 to 6.5 mm range (Table 5). However, none of the measured parameters clearly

distinguished the groups with the complaint of oral malodour from the group with no complaint of malodour.

This suggested that either the criteria that was used to form the groups, i.e., the patients' own self assessment of malodour, was deficient in identifying the individuals with true malodour as measured organoleptically (4), or that the many measured parameters were not contributory to the malodour. The latter possibility is more likely given the repeated demonstration that the overgrowth of bacteria in the coating of the tongue is the main cause of oral malodour (2,6-10). We had included only one question concerning the tongue, and that asked the individuals whether they cleaned their tongues. Most individuals, i.e., 60 to 65%, stated that they cleaned their tongues (Table 4), and again there was no difference between the groups. However, more meaningful measurements would have been an examination of the tongue in which the amount of tongue coating was qualitatively assessed, the presence of deep fissures noted, and BANA tests performed on the coating (see chapter 9). Ideally, organoleptic and objective measurements should be performed.

The present study confirms in an indirect way the importance of the tongue in the generation of oral malodour, i.e., none of more than 50 oral, dental, salivary or medical parameters could be associated with a complaint of oral malodour. This suggests that missing parameters, namely the status of the tongue (10), or other yet to be identified parameters are the main causes of oral malodour. In regard to the latter, one intriguing finding from the medical history was the tendency for patients with a complaint to have a history of allergies. This is interesting in light of Rosenberg's suggestion that postnasal drip on the tongue dorsum is a major source of oral malodour (18).

Acknowledgements

This research was supported by USPH Research grant DE-09142 from the National Institute of Dental Research. Walter Bretz, and Katherine Curry assisted in the collection of the dental data. Yin-Maio Chen provided the statistical analysis, Janice Stoll the bacterial data and Carol Gerlach prepared the tables.

References

1. Anonymous. (1995) Survey conducted at ADA reveals interesting trends. *Dental Economic.* Dec 1995, p6.

2. Miyazaki H., Sakao S., Katoh Y. and Takehara T. (1996) Oral malodor in the general population. In: Rosenberg M. (Ed) *Bad breath: Research perspectives.* Ramot Publ., Tel Aviv Univ., pp 119-136.
3. Loesche W.J., Abrams J., Terpenning M.S., Bretz W.A., Dominguez B.L., Grossman N.S., Hildebrandt G.H., Langmore S.E. and Lopatin D.E. (1995) Dental findings in geriatric populations with diverse medical backgrounds. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 80:43-54.
4. Rosenberg M., Kozlovsky A., Gelernter I., Cherniak O., Gabbay J., Baht R. and Eli I. (1995) Self-estimation of oral malodour. *J Dent Res* 74:1577-1582.
5. Kostelc J.G., Preti G., Zelson P.R., Brauner L. and Baehni P. (1984) Oral odors in early experimental gingivitis. *J Periodont Res* 19:303-312.
6. Yaegaki K. and Sanada K. (1992) Biochemical and clinical factors influencing oral malodor in periodontal patients. *J Periodontol* 63:783-789.
7. Tonzetich J. (1977) Production and origin of oral malodor: a review of mechanisms and methods of analysis. *J Periodontol* 48:13-20.
8. Rosenberg M., Kulkarni G.V., Bosy A. and McCulloch C.A.G. (1991) Reproducibility and sensitivity of oral malodor measurements with a portable sulfide monitor. *J Dent Res* 70:436-440.
9. Bosy A., Kulkarni G.V., Rosenberg M. and McCulloch C.A.G. (1994) Relationship of oral malodor to periodontitis: evidence of independence in discrete subpopulations. *J Periodontol* 65:37-46.
10. De Boever E.H. and Loesche W.J. (1995) Assessing the contribution of anaerobic microflora of the tongue to oral malodor. *J Amer Dent Assoc* 126:1384-1393.
11. Loesche W.J., Bromberg J., Terpenning M.S., Bretz W.A., Dominguez B.L., Grossman N.S., Hartsuff B. and Langmore S.E. (1994) Xerostomia, xerogenic medications and food avoidances in selected geriatric groups. *J Amer Geriatrics Soc* 43:401-407.
12. Miller A.J., Brunelle J.A. and Carlos J.P. (1987) Oral health of United States adults. *National Institute of Health 1987* DHHS pub No. 87-2868.
13. Loesche W.J. (1979) Clinical and microbiological aspects of chemotherapeutic agents used according to the specific plaque hypothesis. *J Dent Res* 58:2404-2459.
14. Silness J. and Löe H. (1964) Periodontal disease in pregnancy: H. correlation between oral hygiene and periodontal condition. *Acta Odontol Scand* 24:747-759.
15. Hildebrandt G.H., Loesche W.J., Lin C-F. and Bretz W.A. (1995) Comparison of the number and type of dental functional units in geriatric

populations with diverse medical backgrounds. *J Prosthetic Dent* 73:253-261.

16. Shem R.J., Fox P.C., Cain J.L. and Li S. (1990) A method for measuring the flow of saliva from the minor salivary glands. *J Dent Res* 69:1146-1149.
17. Loesche W.J., Schork A., Terpenning M.S., Chen Y-M. and Stoll J. (1995) Factors which influence the salivary levels of selected organisms in older individuals. *J Clin Microbiol* 33:2550-2557.
18. Rosenberg M. (1996) Clinical assessment of bad breath: current concepts. *J Am Dent Assoc* 127:475-482.

A study on the Relationship between Oral Parameters and Breath Odour

*H. Onozawa, T. Yasui and S-I. Nakao**

Introduction

The purpose of this study was to clarify the relationship between oral malodour and some oral environmental factors. Salivary occult bleeding level, oral hygiene index (OHI) and the number of missing teeth were selected as local environmental factors, and related to the complaint of malodour.

Subjects

Three hundred and thirty-nine residents (ranging in age from 40 to 60) who live in Nerima, Tokyo participated in this study. Subjects were divided into two groups whether having a complaint of oral malodour or not. The prevelance according to the age categories can be found in Table 1.

Table 1 : Distribution of subjects per age category and gender

Age category	Male	Female	Total
40-44	17	44	61
45-49	10	49	59
50-54	11	45	56
55-59	21	57	78
> 60	23	62	85

Evaluation of salivary occult bleeding

The evaluation of occult blood in saliva was measured with a test strip (Sahvaster-Bld®, Showa Yakuhin Kako, Co. Ltd., Tokyo, Japan). This test strip contains cumene hydroperoxide, toluidine, and a buffering agent. The color changes from yellow to dark green according to the salivary occult

* H. Onozawa, T. Yasui and S. Nakao, Department of Dental Health and Preventive Dentistry, Meikai University School of Dentistry, Sakado, Saitama 350-02, Japan.

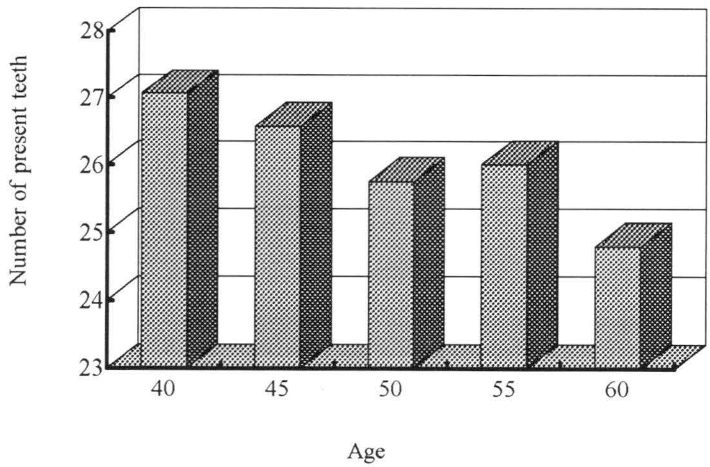


Fig. 1 : Results of salivary occult bleeding test in the subjects with complaints of oral malodour.

bleeding level, and can be classified into four levels by a standard color chart. The relationship between oral malodour and salivary occult bleeding level was shown in Fig. 1.

Subjects in the complaint group had a significantly higher percentage of salivary occult bleeding level ($p < 0.05$; chi-square test). This difference was observed especially under the age of 55 years old.

Assessment of oral hygiene level

For the assessment of oral hygiene condition, an oral hygiene index (OHI) was used. Dental plaque was disclosed with disclosing agent (5ml, 2% solution of erythrosine). The debris index (DI) was also evaluated. After brushing, the calculus index (CI) was assessed. OHI was calculated as the sum of the DI and the CI. The OHI of the different age categories can be found in Fig. 2.

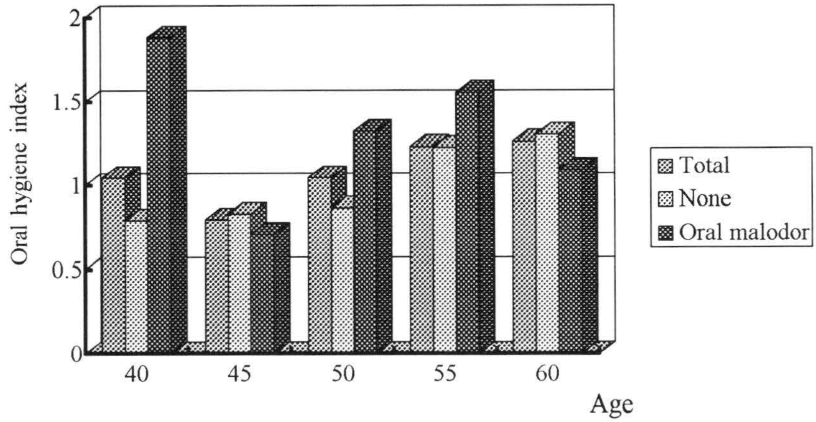


Fig. 2 : Comparison of OHI by complaint of oral malodour.

Evaluation of dentition

The average number of missing teeth was 0.9, 1.4, 2.2, 2.0 and 3.2 for the group of 40, 45, 50, 55 and 60 years respectively (Fig. 3)

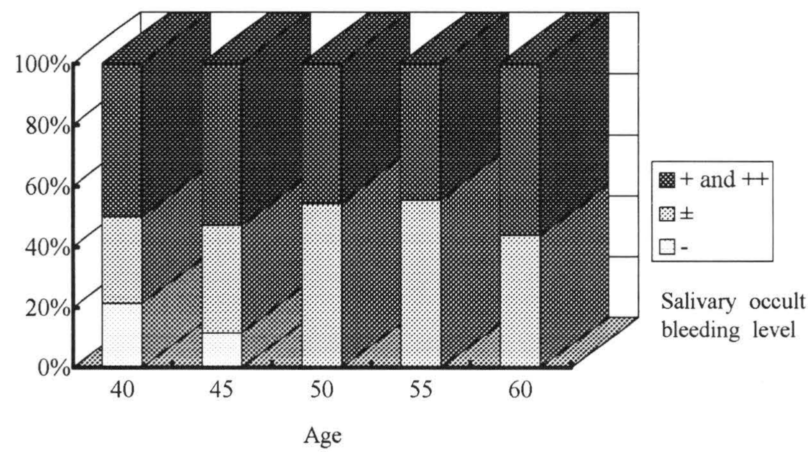


Fig. 3 : The number of teeth present by age.

Conclusion

We examined some selected oral environmental factors in relation to oral malodour. The prevalence of oral malodour gradually increased with age up to 50 years old. In groups over 55 years old, however, the prevalence of oral malodour decreased. The results demonstrated that the salivary occult bleeding level was highly related to oral malodour.

Experiences of a Belgian Multidisciplinary Breath Odour Clinic

G. Delanghe, J. Ghyselen, L. Feenstra and D. van Steenberghe[*]

Introduction

Oral malodour is a frequent problem. Over half of the population complains of a noticeable malodour in the morning. It causes embarrassment, frustration and despair and often leads toward social and professional isolation and marital problems. The malodour can be camouflaged in many ways, some of which are strongly promoted by the news media. However it should be remembered that the malodour is a symptom, thus the cause should rather be treated. A multidisciplinary approach (periodontology, ENT, internal medicine, psychiatry) is a good way to do so.

Two years ago a multidisciplinary consultation on oral malodour was initiated at the University Hospital in Leuven. As far as we know, no such consultation exists in Belgium or even in Europe. At the start some publications in national journals and interviews in the media proved helpful to inform generalists (both physicians and dentists) and patients of this new activity. The frequency of the consultation had to be increased from once a month to once a week.

Material and Methods

The consultation takes place at the E.N.T. department. Patients are seated in the same waiting room as other E.N.T. patients to ensure anonimity. They are examined by an E.N.T. specialist and a periodontologist. A psychologist and internist can be called for advice.

History taking consists of four parts. Specific questions on the oral malodour itself are asked (duration, evolution, daily pattern of the complaint, whether the complaint is made by the patient or others and description of the malodour). Questions are asked about habits (smoking, drinking, medication and diet) and systemic diseases (lungs, stomach, liver, kidney and pancreas). An E.N.T. history is taken (nasal obstruction, nasal discharge, postnasal drip,

---

* G. Delanghe*, J. Ghyselen°, L. Feenstra* and D. van Steenberghe°, * Department of ENT, ° Department of Periodontology, Faculty of Medicine, Catholic University Leuven, Capucijnenvoer 7, 3000 Leuven, Belgium.

allergy, tonsil problems, dysphagia, voice problems and previous E.N.T. encounters). The history of the oral cavity is recorded (frequency of tooth brushing, interdental cleaning, tongue brushing, prosthesis, frequency to visits at the dentist).

The clinical examination consists of three parts. First, organoleptic breath evaluations, one at rest and one during speech (1) are performed by two independent examiners. A score from 0 to 3 is given (0 = no oral malodour, 1= mild oral malodour, 2= moderate oral malodour, 3= severe oral malodour). Patients are asked to lick the back of their hand (2). An organoleptic evaluation of this sample and of a scraped sample of the backside of the tongue is also done. Next the nose, nasopharynx, throat, larynx and neck are examined. All patients mentioning nasal problems are examined by nasal endoscopy. If tonsils are cryptic an organoleptic evaluation is performed on a sample of tonsillar excretions taken with a cotton swab. Finally the oral cavity is inspected. Attention is paid to caries, bacterial plaque, gingival inflammation, periodontal pockets, prosthesis and tongue coating. The latter was scored according to the surface involved as 0 = no coating, 1 = thin coating on 1/3 of the tongue, 2 = thin coating on 2/3 of the tongue or thick coating on 1/3 of the tongue, 3 = thick coating on 2/3 of the tongue. An objective evaluation of the amount (ppb) of volatile sulphides (hydrogen sulphide, H_2S, methyl mercaptan, CH_3SH, and dimethyl sulphide, $(CH_3)_2S$) in the patients' breath is made by the Halimeter (Interscan Corporation, model RH-17E, Chatsworth, USA) (3-5).

If the clinical examination of the E.N.T. and oral region is negative and the patient complains about gastric problems, a ^{13}C-urea test is proposed, to detect *Helicobacter pylori*. The correlation between halitosis and *H. pylori* has been suggested by several authors but remains very dubious (6,7).

Results

Thus far 260 patients visited our outpatient breath odour clinic. They came from all over the country. Five patients (2%) were foreigners (France 2, Luxemburg 2, The Netherlands 1). Two hunderd and nineteen patients (84%) came spontaneously to the consultation. Nineteen (7%) were referred by a general physician, 14 patients (5%) were referred by another department of the University Hospital, 6 patients (3%) from another hospital and 2 (1%) by a dentist. All of the patients had been looking for help elsewhere.

There was no gender predominance; 48% males (n = 125) and 52% females (n=135). The age range was 2 to 77 years, with a mean of 36 years (SD=13.5).

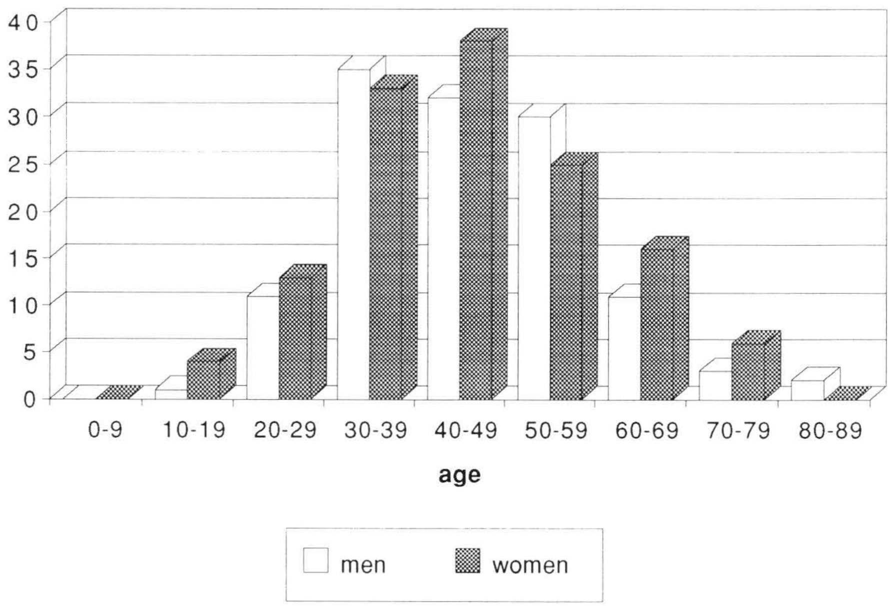


Fig. 1 : Age and gender distribution.

(Fig. 1) There was no significant difference in age distribution between men and women (x^2-test; p=0.5). All patients belonged to the upper and middle socio-economic class, which was based on their educational/occupational background as reported in the questionnaire.

Ten of our patients (4%) had only recent complaints of oral malodour (1 year). Eighty-four patients (32%) had complaints for more than 1 but less than 5 years. One hundred and sixteen patients (64%) had complaints for more than 5 years. A x^2-test showed no significant difference ($p > 0,5$) in duration of the complaint between men and women. One hundred and fifty-one of our patients (58%) had not noticed the malodour themselves, but were informed by others of their bad breath. Sixty-two patients (24%) were told by others and noticed it themselves as well. Only 47 patients (18%) noticed the malodour themselves, without being informed by others. (Fig. 2) No significant difference was found between men and women (x^2-test; $0.5 > p > 0.1$).

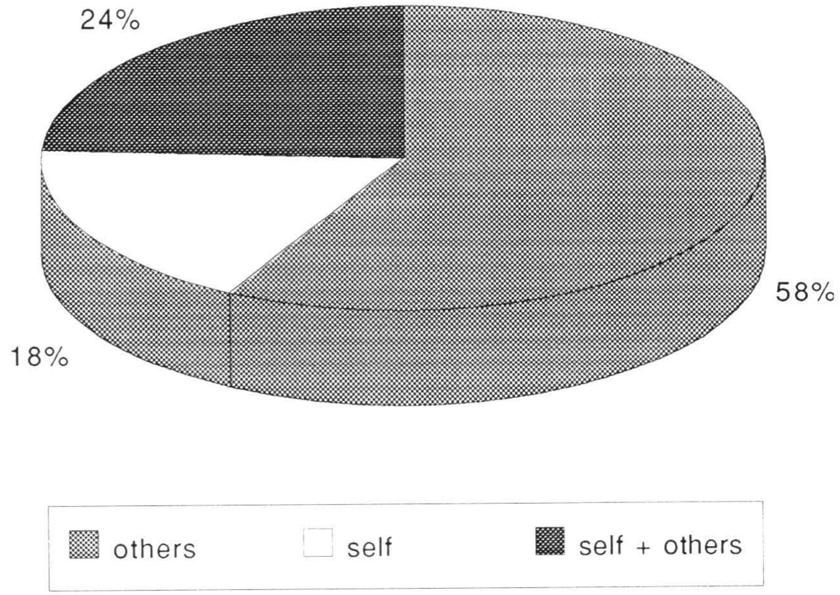


Fig. 2 : Source of complaining about the malodour.

Seventeen percent (n = 44) of the patients were smokers. None of the patients admitted to have an excessive drinking habit. Only 5% (n = 12) followed a diet, such as salt-free, vegetarian, low-fat or lactose-free. Only one patient claimed a decrease of the malodour intensity after starting a lactose-free diet. No patient with halitosis caused by a systemic disease was found among our patients.

Two hunderd and twenty-five patients (87 %) were deemed to have an oral cause of their halitosis, 21 (8%) had a cause in the E.N.T. region and for 14 patients (5%) no cause could be identified. (Fig. 3) The organoleptic evaluation of the breath of those last patients was degree 0 for nine of them, degree 1 for four patients and degree 2 for one patient. The range of their objective evaluation by the Halimeter was 50 to 188 ppb, with a mean of 97 ppb, which was significantly lower than the mean halimeter value of the entire group of patients ($p < 0.01$). Twelve of the patients were women. (Table 1) A few patients were advised to have a psychiatric consultation. All of them refused.

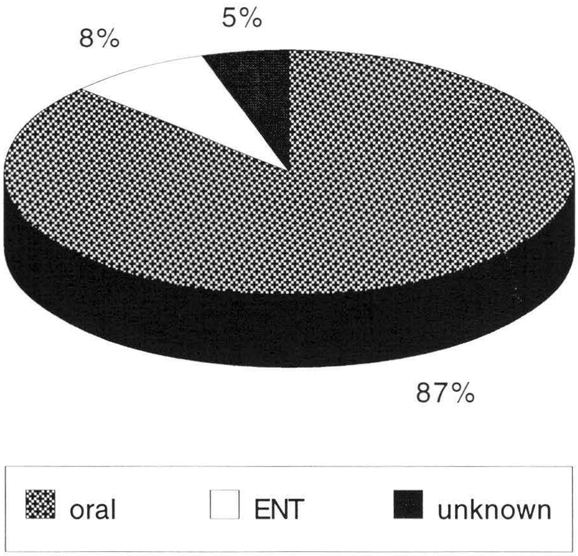


Fig. 3 : Cause of malodour.

Table 1 : List of patients who had no objective cause for their malodour

n	sex	age	organoleptic evaluation	Halimeter value (ppb)
1	m	25	1	124
2	m	34	0	56
3	f	33	1	132
4	f	39	1	82
5	f	45	0	54
6	f	24	0	97
7	f	37	1	86
8	f	56	2	86
9	f	28	0	98
10	f	50	0	129
11	f	22	0	73
12	f	16	0	50
13	f	23	0	106
14	f	39	0	188

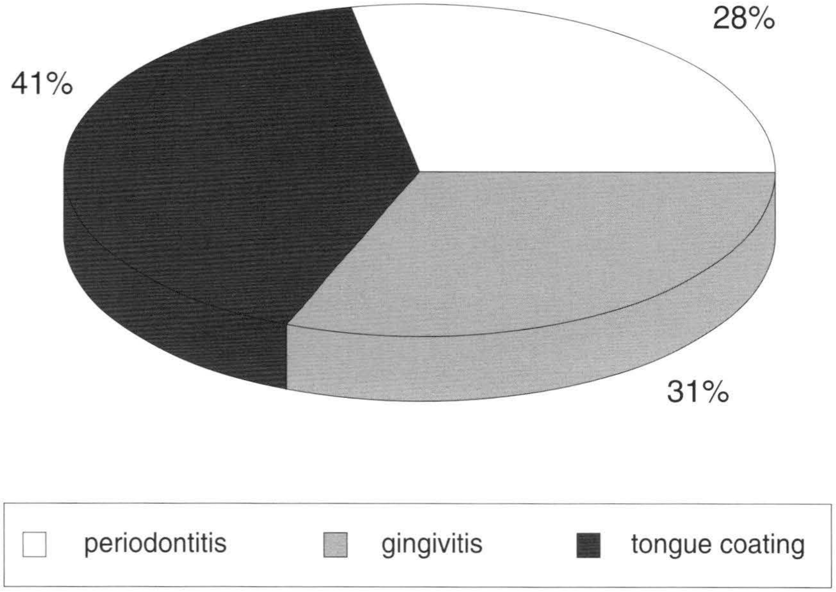


Fig. 4 : Oral causes of malodour.

In the group of patients with an oral cause 41% (n = 92) had tongue coating, 31% (n = 70) had gingivitis and 28% (n = 63) had periodontitis. (Fig. 4) Patients were advised to improve their oral hygiene habits (teeth, interdental spaces and tongue) or to visit their dentist or periodontologist for treatment.

In the group of patients with ENT problems fifteen suffered from chronic tonsillitis, four from chronic sinusitis, one had ozaena and one had a foreign body (piece of paper) in his nose. (Fig. 5) According to their pathology, patients were advised to undergo either a tonsillectomy, functional endoscopic sinus surgery or a local nasal treatment. Before tonsillectomy was executed a perfect oral hygiene was demanded. There was no significant difference in distribution of the organoleptic score between patients with chronic tonsillitis and the rest of the patients (X^2-test; $p > 0.1$).

The organoleptic evaluation of the breath was significantly correlated with the objective measurement by the Halimeter ($r = 0.2731$; $p < 0.001$). The Halimeter values were correlated with the degree of tongue coating ($r = 0.1913$; $p < 0.01$) and the depths of the gingival pockets ($r = 0.2156$; $p < 0.001$). No significant correlation was found between Halimeter values and degree of malodour of tonsillar excretions ($r = 0.0880$; $p > 0.5$). A significant correlation was found between the degree of tongue coating and

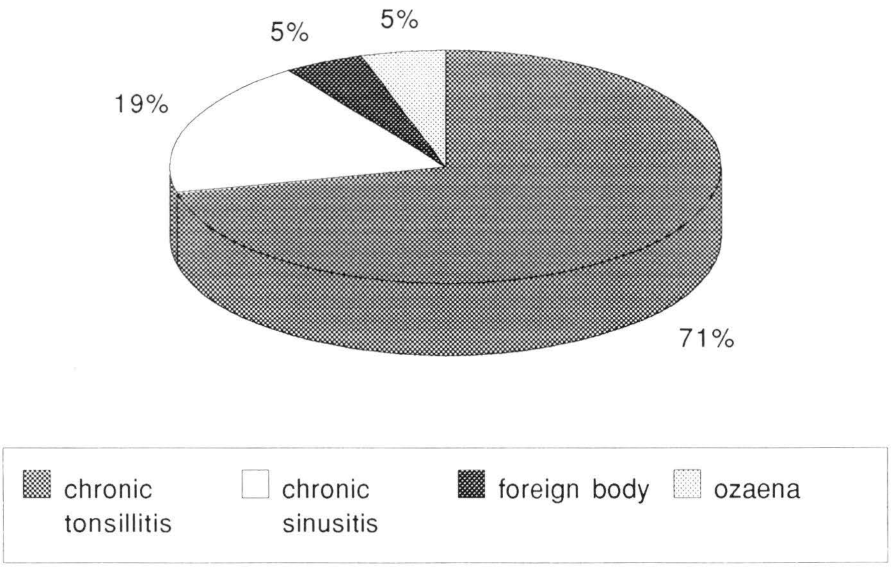


Fig. 5 : ENT causes of malodour.

the organoleptic evaluation of this coating (r = 0.5481; $p < 0.001$).

Twenty-one C_{13} - urea-test were performed. Seven (33,3 %) of them were positive. This is not unlike the percentage of positive tests found in the "normal" European population. Three patients agreed to start the expensive treatment, a combination treatment of amoxicillin 2 g/day, metronidazole 1,5 g/day and omeprazole 20 mg/day. They got all cured from their *Helicobacter pylori*, but none noticed a decrease of the oral malodour.

Only ninety-three (36%) of our patients were seen for a follow-up consultation after treatment i.e., two to six months after the initial visit. Fifty-five of them (59%) reported disappearance of malodour complains while 14 (15%) reported that the environment did not complain anymore, although they themselves noticed malodour from time to time. Eleven patients (12%) reported a decrease of the malodour, but their environment still noticed the odour too. Thirteen patients (14%) noted no improvement at all. There was no significant difference in the distribution between men and women (x^2-test; p = 0.2). Some of the results were received by phone, so no organoleptic or Halimeter measurement could be performed for these patients. For this no correct statistical figures can be given concerning subjective evaluation by the patient versus organoleptic and Halimeter value.

Discussion

After two years of the breath odour clinic start-up 260 patients were examined by a multidisciplinary team, consisting of an ENT specialist, a periodontologist and occasionally a specialist in internal medicine. A psychiatrist was on call but no patient accepted this. The patient population showed no gender preference. Rosenberg and Leib (8) found in their population a predominance of 60% female patients. The age distribution is from 2 to 77 years old, with a mean age of 36 years. The characteristic patient is a person who complains of his oral malodour for more than 5 years, has been looking for help at several places and belongs to the middle or upper socio-economical class. Fifty-eight percent of the patients has not noticed the malodour themselves, but were informed by others. Describing the characteristics of the malodour turned out to be very difficult or even impossible for most of the patients. Seventeen percent of the patients are smokers. As told by many of our patients, tobacco smell is a common accepted breath smell and therefore often used to mask the problem of oral malodour. Only 5 % of the patients followed a diet. One patient claimed to have a decrease of the malodour after starting a lactose-free diet.

Most frequently (87%) the oral malodour is caused by an oral problem (tongue coating > gingivitis > periodontitis). These results are comparable with those described by Rosenberg and Leib (8). In 8 % of the patients ENT pathology is found as cause of the malodour (chronic tonsillitis, chronic sinusitis, ozaena and foreign body in the nose). For 5 % of the patients no cause at all could be found. Seventy-five % of those patients had an objective measurement by the Halimeter in the normal range (< 150 ppb) and also a complete negative organoleptic evaluation. Psychiatric advice was proposed at that moment. All refused. One third of the ^{13}C-tests, to diagnose *H. pylori*, was positive. Three patients were treated and got cured of their *Helicobacter*, but their own perception and our organoleptic evaluation of oral malodour persisted. Therefore no conclusion whatsoever could be drawn.

The organoleptic evaluation of the breath, done by two independent and trained persons, is significantly correlated with the objective measurement by the Halimeter. Some of our patients had a clear positive organoleptic evaluation with a negative objective evaluation by the Halimeter. This confirms that other volatile elements than volatile sulphur compounds can be responsible for oral malodour such as indole. Halimeter values appeared significantly correlated with the degree of tongue coating and the depth of the gingival pockets. A strong correlation was found between the degree of tongue coating and organoleptic evaluation of this coating. There was no significant correlation between the odour of tonsillar excretions and Halimeter

values. No significant difference was found in distribution of the organoleptic score between the patients with tonsillar problems and the rest of the patients. Rosenberg (1) has proposed that tonsilloliths contribute primarily to self conception of bad breath, rather than actual oral malodour.

Up till now only 36 % of the patients were seen in a follow-up consultation. The majority (59 %) was cured. Fourteen percent showed no improvement at all.

Conclusion

Oral malodour is a frequent problem, which benefits from a multidisciplinary approach (periodontology, ENT, internal medicine, psychiatry). This can save time and avoid the risk of concluding too quickly that an evident symptom, e.g. gingivitis is the cause of the reported malodour, while a more cryptic etiology is overlooked. History taking should focus on the odour itself, oral hygiene habits, ENT pathology, systemic pathology and general habits of the patients. During clinical examination the organoleptic evaluation of the breath (at rest and during speech), the tongue coating and the tonsillar excretions, performed by trained people, is of great importance. An objective measurement of the amount of volatile sulphur compounds in the patients breath, by the Halimeter, is very useful and easy to perform, although one should be aware of its limitations. Oral malodour has no sex- or age-predominance. Typical to the problem is the "bad breath paradox"(8). Often, people suffering from bad breath remain completely unaware of their own oral emission, whereas others remain convinced that they suffer from oral malodour, although no objective basis could be found. Tongue coating is the most frequent cause of oral malodour. Initial treatment strategies should focus on oral hygiene i.e., tooth brushing, cleaning of the posterior tongue dorsum, interdental cleaning, visit to the dentist or periodontologist. In a few cases tonsillectomy or endoscopic sinus surgery may be necessary.

References

1. Rosenberg M. (1996) Clinical Assessment of Bad Breath : Current concepts. *J Am Dent Assoc* 127: 475-482.
2. Kozlovsky A., Gordon D., Gelernter I., Loesche W.J. and Rosenberg M. (1994) Correlation between the BANA-test and oral malodour parameters. *J Dent Res* 73:1036-1042.

3. Rosenberg M., Kulkarni G.V., Bosy A. and McCulloch C.A.G. (1991) Reproducibility and sensivity of oral malodour measurements with a portable sulphide monitor. *J Dent Res* 70:1436-1440.
4. Rosenberg M. and McCulloch C.A.G. (1992) Measurements of oral malodour: current methods and future prospects. *J Periodontol* 63:776-782.
5. Yaegaki K. and Sanada K. (1992) Volatile sulphur compounds in mouth air from clinically healthy subjects and patients with periodontal disease. *J Periodont Res* 27:233-238.
6. Norfleet R.G. (1993) Helicobacter halitosis. *J Clin Gastroenterol* 16:274.
   Tiomny E., Arber N., Moshkowitz M., Peled Y. and Gilat T. (1992) Halitosis and *Helicobacter pylori*. *J Clin Gastroenterol* 15:236-237.
7. Rosenberg M. and Leib E. (1995) Experiences of an Israeli Malodour Clinic. In : Rosenberg M. (Ed) *Bad Breath : Research perspectives*. Ramot Publishing, Tel-Aviv University, Tel-Aviv; Israel, pp137-148.
8. Rosenberg M., Kozlovsky A., Gelernter I., Cherniak O., Gabbay J., Baht R. and Eli I. (1995) Self-estimation of oral malodour. *J Dent Res* 74:1577-1582.

A Simple Detector for Oral Malodour

*E. Maita**

Halitosis is a symptom encountered in gingivitis, periodontitis, extensive dental caries or poor oral hygiene. The substances related to bad breath are volatile sulphur-containing compounds, such as methyl mercaptan and dimethyl sulphide. A tindioxide semiconductor gas sensor sensitive to reduce gases was tested. Both methyl mercaptan and hydrogen sulphide gases showed linear relationships between the sensor resistance and the gas concentration in a range between 0.3 and 3 ppm. In contrast to these gases, ethyl alcohol gas had lower sensitivity (1/30). The replaceable charcoal filter had remarkable effectiveness for removing the background drifts.

In a group of thirty-two patients, no significant linear relationship was observed between the concentrations of reductive gases and the plaque score. In eight volunteers who stopped tooth brushing for 24 hours to allow accumulation of dental plaque, five minutes brushing led to a significant reduction of the gas concentration.

Thirty-five disabled patients had higher gas concentration than four healthy subjects. The patients manifesting drooling or gingival hyperplasia had higher gas concentrations than control patients.

The BB checker used for this investigation was simple and easy to use. It could be useful to monitor the oral hygiene instruction.

Introduction

Recently, the number of patients complaining of halitosis is increasing in the dental office. Halitosis is a symptom encountered with gingivitis, periodontitis, extensive dental caries or poor oral hygiene (1). The substances that cause bad breath are ammonia, amines, thiol compounds, indole, skatole or especially, volatile sulphur-containing compounds (VSC), such as methyl mercaptan and dimethyl sulphide (2).

The gas chromatography technique has been normally adopted for the experimental studies on malodour (3). Since it is unconvenient to use at chair

---

* E. Maita, Department of Endodontics and Periodontics, School of Dentistry, Tohoku University, 4-1 Seiryo-machi 980 Sendai, Japan.

side, some simpler device is desirable. A tin dioxide semiconductor gas sensor which is sensitive to reductive gases, has been developed by Tokuyala Corp., Tokyo, Japan (the BB checker™). The aim of the present study is to estimate the detection threshold for the reductive gases with this device.

Material and Methods

Oral Gas Detector

"BB checker" (Tokuyama Corp. Tokyo, Japan) has a tin dioxide semiconductor gas sensor and is sensitive to reductive gases (Fig. 1). It consists of a sensor probe and a main body. Oral gases are automatically inhaled into the sensor by the probe. The sensor probe has also a replaceable charcoal filter in order to remove the background gases.
The principle of measurement of the reductive gases by the sensor is shown

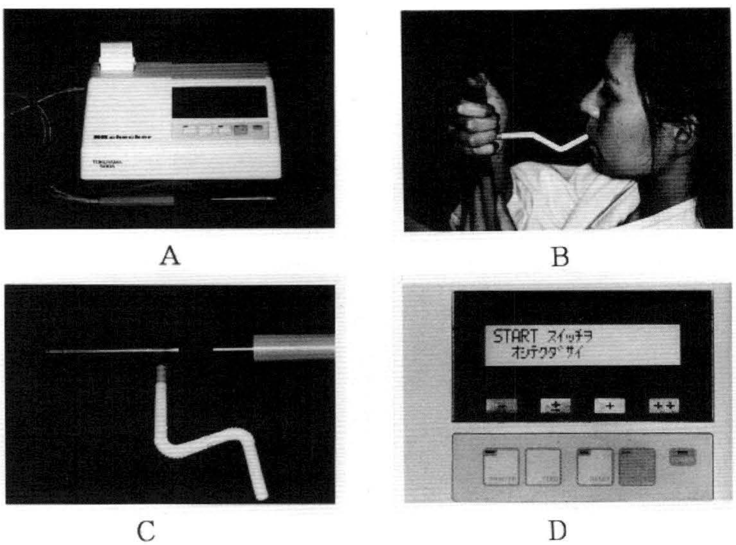
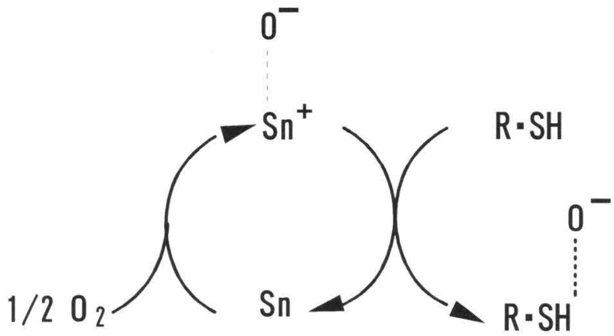


Fig. 1 : A : BB checker
B : When a patient pushes the button the oral gases are inhaled automatically into sensor portion.
C : Probe portion with disposable tube
D : Main body



$$1/2\ O_2 + (SnO_2) \longrightarrow O^- \cdot (SnO_2)$$

$$R^- \cdot SH + O \cdot (SnO_2) \longrightarrow O \cdot R^- \cdot SH + (SnO_2)$$

Fig. 2 : Reaction of reductive gases to tin oxide semiconductor.

in Fig. 2. The semiconductor sensor is an amorphous tin oxide membrane of 0.1 µm thickness. The relative electric resistance of reductive gases to background gases is displayed as nmol on the main body.

Patients

1. 32 patients visiting the department of periodontology were sampled at random for estimating the relationship between plaque scores and the concentration of oral gases.
2. 8 young healthy students volunteered to stop oral hygiene measures for 24 hours to allow bacterial plaque to accumulate.
3. 35 mentally disabled patients with or without drooling, gingival hyperplasia or mouth breathing were sampled at Nishitaga Hospital for oral gas concentration measurement.

Results

Detection capacity with the BB checker™ (Fig. 3)

Both methylmercaptan and hydrogen sulphide used for the calibration, showed linear relationships between 0.3 and 3 ppm. In contrast to these gases, the sensor showed depressed sensitivity, (one thirtieth) for ethyl alcohol gas.

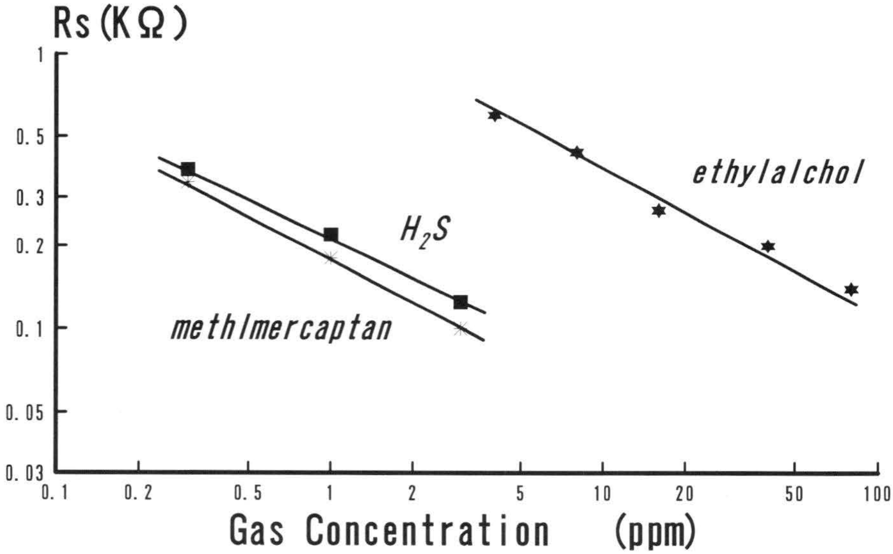


Fig. 3 : Measurement of the authentic gases.

Removal of outer gases by a packed charcoal filter (Fig. 4)

Ethyl alcohol gas (5 ppm) was injected in the gas sampling bag and lead to the detector via disposable tube. The gas concentration was measured in the gas atmosphere (2 ppm) with or without the filter. Measured after removing the filter, the gas (5 ppm) measured only 2.1 ppm. However, when the filter was repacked into the probe, the detector showed the original concentration of 5 ppm.

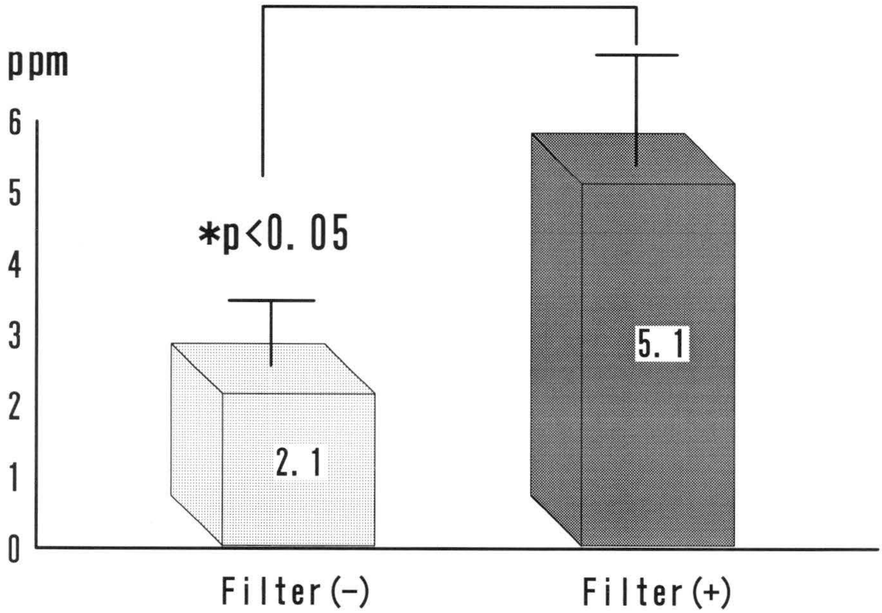


Fig. 4 : Effect of replaceable charcoal filter.

Relationship between oral gas concentration and plaque score (Fig. 5)

Oral gas concentration increased with plaque score, however, there was no statistical significant relationship.

Change of oral gas concentration by tooth brushing (Fig. 6)

Gas concentration gradually eliminated to the lowest level (0.1 ppm) at five minutes of the brushing time ($p < 0.05$).

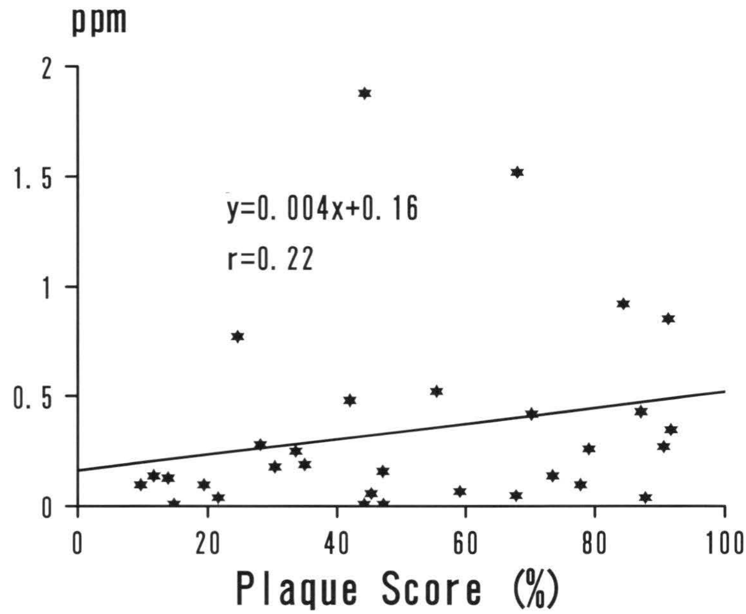


Fig. 5 : Relationship between the oral gas concentration and plaque score in 32 patients.

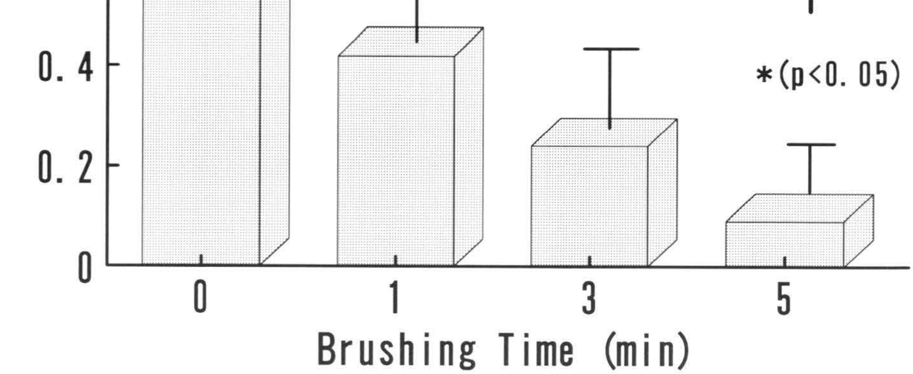


Fig. 6 : Effect of toothbrushing on the mouth gas concentration.

Oral gas concentration in handicapped patients

The disabled patients demonstrated higher gas concentration than the normal subjects ($p < 0.01$) (Table 1). The patients with drooling or gingival overgrowth had significantly higher gas concentration that the patients without these symptoms ($p < 0.01$) (Fig. 7). However, there was no difference between the patients with and without mouth breathing.

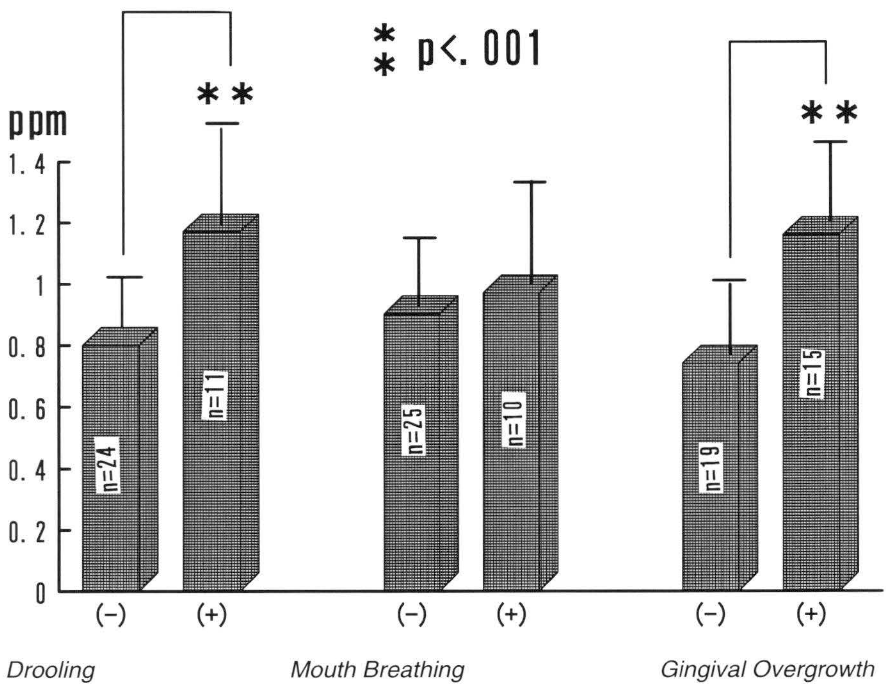


Fig. 7 : Oral gas concentration of handicapped patients with or without clinical signs.

Table 1 : Measurement of concentration of oral gases in disabled patients

Subjects	ppm	
Healthy (n= 4)	0.06 ± 1.04	** $p < 0.01$
Disabled (n=35)	0.92 ± 1.09	

Disabled patients had significantly higher gas concentration than the normal subjects ($p < 0.01$).

Discussion

BB checker™ has a compact feature (1.7 kg in weight and 22 x 16.6 cm width and 8.6 cm height). Since the gas is inhaled automatically via a disposable tube into the sensor probe portion, it is not necessary to collect the oral gas into a gas sampling bag as in gas chromatography procedures. The gas level is displayed on the monitor as ppm values. Unrelated gases in the atmosphere are removed by the charcoal filter.

Although, the data did not reach significance levels, the oral gas concentration tended to become higher with higher plaque scores.

The measurement decreased significantly after 5 min brushing in volunteers who had accumulated plaque for 24 hours. As pointed out by Sulser (4), 47% of patients complaining about halitosis can be related to their oral condition. Thus, the improvement of the oral condition by cleansing procedures as reported by Tonzetich (2) can eliminate the malodour.

Patients with psychogenic halitosis also often visit the dental clinics. Therefore, this device could be useful to discriminate the psychogenic patients by demonstrating them the objective levels at the chairside.

References

1. Lu D.P. (1982) Halitosis: an etiologic classification, a treatment approach, and prevention. *Oral Surg.* 54:521-526.
2. Tonzetich J. and Ng S.K. (1976) Reduction of malodor by oral cleansing procedures. *Oral Surg, Oral Med Oral Pathol* 42:172-181.
3. Tonzetich J. (1971) Direct gas chromatographic analysis of sulphur compounds in mouth air in man. *Archs oral Biol* 16:587-597.
4. Sulser G.F., Brening R.H. and Fosdick L.S. (1939) Some conditions that affect the odor concentration of the breath. *J Dent Res* 18:355-359.

Attitudes towards One's and Others' Oral Malodour

K. Yaegaki, H. Matudaira, S. Sano and T. Kitamura[*]

Introduction

Recently, Japanese people have paid more attention to bad breath and oral hygiene than in former times (1). In particular, the younger generation is interested in preventing oral malodour. For the past decade the marketing of mouthwash, tongue brushes and tongue scrapers has dramatically increased in Japan. For many years the oral hygiene of the Japanese population had lagged behind the people of other developed countries. The Japanese economy, however, has increased over the past two decades, and consequently, Japanese society has changed. For example, the lifestyles and eating habits have been steadily westernized, and the quality of life has been dramatically improved. During these changes of Japanese society, the dental hygiene of the population has improved. We speculate that the changes of Japanese society have stimulated more interest in oral hygiene and bad breath.

The number of halitosis cases with psychosomatic or psychiatric aspects has been increasing during the past two decades (2-5). The biochemical pathology of oral malodour has been elucidated over the years by Tonzetich (6-11), Preti (12), Johnson (13), Ng and Tonzetich (14), Kaizu (15) and Yaegaki (1-3, 16-23). However, it is not simple to treat psychosomatic halitosis such as "halitophobia" (3, 24, 2, 4, 5).

Many difficult cases of psychosomatic halitosis have been reported; even some patients have committed suicide because of their oral malodour (3). All halitosis patients with a psychosomatic component are concerned of other people's behaviour toward their own breath. For example, most patients complained "When I face other people, they cover their nose with the hand because of my bad breath." Sometimes the patients said "They avert their faces," or "They open the window because of my bad breath." Thus, halitosis patients are afraid of other people's attitudes and behaviours toward their oral malodour. Numerous papers of psychosomatic halitosis or psychosis concerned with halitosis have been published in Japan since 1974 (2, 3). However, it is not yet clear whether the numbers of people concerned with

* K. Yaegaki, H. Matudaira, S. Sano and T. Kitamura, Department of Preventive Dentistry, School of Dental Medicine, Tsurumi University, Yokohama, Japan.

other people's bad breath are what the halitosis patients expect, and whether gestures, such as covering one's nose, constitude a reaction for a person's malodour as the patients believe. The purpose of this chapter is to clarify the behaviour toward oral malodour, which is essential to comprehending, consulting, and treating the patients. A detailed account of this work can be found in Yaegaki et al (1).

Table 1 : Subjects for Investigation

Age	Number	%
18y	842	35.2
19y	864	36.2
20y	292	12.2
21y	299	12.5
22y	92	3.9

Table 2 : Investigated Matters

A. Subjective symptoms of gingivitis
1. Gingival bleeding caused by toothbrushing
2. Gingival bleeding caused by eating
3. Gingival swelling
4. Gingival pain
5. Ill defined pain in gingiva

B. Discomfort in the oral cavity
1. Early morning
2. Other times

C. Own oral malodour
1. Using prevention
2. Behaviours
   - Covering the mouth with the hand
   - Keeping distance from another person
   - Others

D. Other people's oral malodour
1. Covering the noses with the hand in public transportation
2. Averting the face in public transportation
3. Covering the noses with the hand when facing another person
4. Averting the face when facing another person
5. Others

Materials and Methods

The preliminary investigation suggested that the younger generation of female Japanese had more interest in oral malodour than other female generations or the male population (1). Therefore, 2389 female volunteers of 18-26 years old were surveyed to clarify their attitude and behaviours toward oral malodour (Table 1). Dentists, physicians, nurses, oral hygienists, paramedics and students in such health related fields were excluded from the study to avoid possible bias. The summary of the questionnaire, which is employed in this study, is shown in Table 2. As it has been clearly demonstrated that gingival pathology contributes to pathological malodour (9, 19, 21), the subjective symptoms of gingivitis or periodontitis were evaluated. Since the putrefactive activity of saliva and cellular elements in the saliva cause oral malodour (11, 16-18), a subject's perception of discomfort in the oral cavity was also included in the questionnaire. The questions regarding the behaviours toward oral malodour have been selected from the complaints or experiences of the halitosis patients in our clinic. The survey has been performed on April 18-22, 1994 in Yokohama, Japan. The results were analyzed by the statistical program package "HALBAU," and the significance was determined by chi-square test.

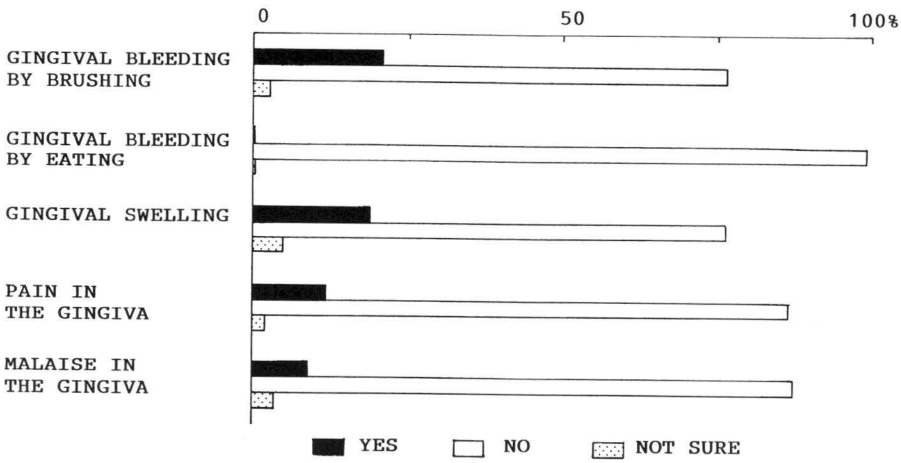


Fig. 1 : Subjective symptoms of gingivitis or periodontitis were investigated in the 2389 subjects. Gingival bleeding when brushing teeth was found in 20.8% of the subjects, but bleeding by eating was found in only 0.3%. Gingival swelling, pain in the gingiva and gingival discomfort (malaise) were noticed in 18.8%, 11.7% and 9.1 %, respectively.

Results

Gingival symptoms

Gingival bleeding, gingival swelling, pain in the gingiva and gingival discomfort were evaluated. We found that 20.8% of the subjects reported gingival bleeding on toothbrushing, while bleeding during or after eating was reported in only 0.3% of the subjects. Gingival swelling, gingival pain and gingival discomfort were found in 18.8%, 11.7% and 9.1 %, respectively (Fig. 1). It was noted that 36.8% of the patients (878 subjects) involved one or more numbers of gingival symptoms as shown in Table 3. Symptoms accompanying gingival bleeding were also surveyed. We found a significant relationship between gingival bleeding by brushing and other gingival symptoms (p.001), but gingival swelling was frequently accompanied by gingival bleeding rather than other gingival symptoms. (i.e., 41.1 % of the subjects with gingival bleeding also had gingival swelling) (Table 4-5)

Table 3 : The number of subjective symptoms

N° of symptoms	N° of subjects	%
1	501	21.0
2	225	9.4
3	110	4.6
4	40	1.7
5	2	0.1
Total	878	36.8

Table 4 : The symptoms related to oral malodour and the subjective symptoms of gingivitis

	Gingival bleeding by brushing teeth n = 456		Malaise in the oral cavity in early morning n = 504		Malaise in the oral cavity in other times n = 129	
Gingival bleeding by brushing teeth	-	-	168*	33.3%	44*	34.1%
Gingival swelling	187*	41.1%	139*	27.6%	34*	26.4%
Pain in the gingiva	118*	25.9%	99*	19.6%	29*	22.5%
Gingival discomfort	95*	20.8%	88	17.5%	39*	30.2%

* $p < 0.001$

Table 5 : Oral malodour and subjective symptoms of gingivitis

	Concern about own malodour		Bothered by other people's oral malodour	
Gingival bleeding at brushing	138*	30.3%	249*	24.4%
Gingival swelling	115*	25.2%	214*	21.0%
Gingival bleeding at eating	2	0.4%	4	0.4%
Gingival pain	82*	18.0%	123	12.1%
Gingival discomfort	73*	16.0%	117*	11.5%
Oral cavity discomfort in early morning	173*	37.9%	265*	26.0%
Bothered by other people's oral malodour	267*	58.6%	-	-
Concerned about own malodour	-	-	267*	26.2%
Total	456		1019	

* $p < .001$

Other symptoms possibly related with oral malodour

Many patients complained that they found malaise of the oral cavity when they recognized their own bad breath. The results indicated that 21.1% of the subjects reported oral cavity discomfort when they got up in the morning, but only 5.4% found it during the daytime (Fig. 2). A significant relationship was found between discomfort at morning and during daytime ($p < 0.001$).

We found a significant relationship between the discomfort during early morning and all other gingival symptoms ($p < 0.001$). The malaise during the daytime was also related to gingival symptoms.

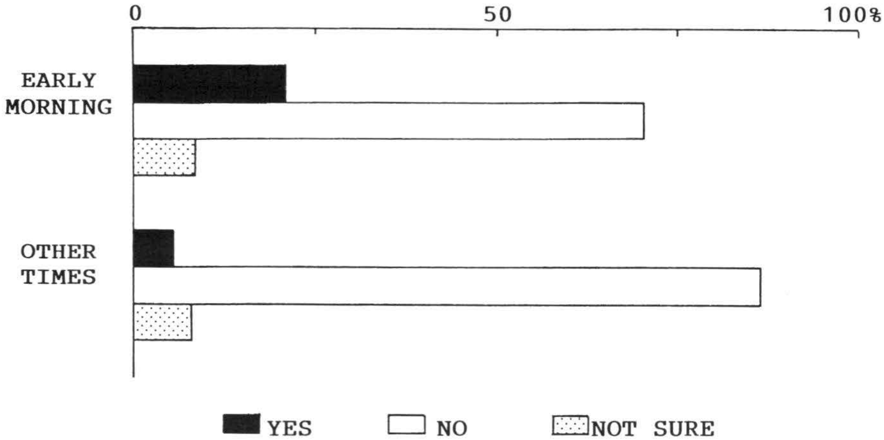


Fig.2 : When awakening, 21.1% of the subjects (n=2389) oral discomfort, but only 5.4% experienced it during the daytime.

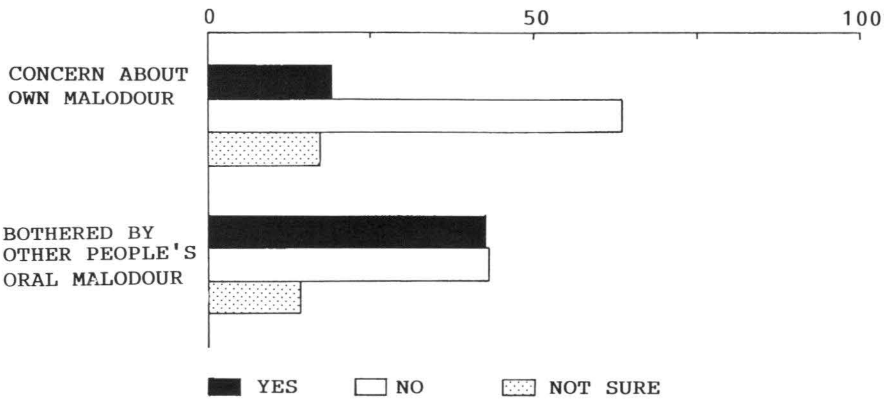


Fig. 3 : Only 19.1 % were conscious of their own oral malodour, but 42.7% (1019 subjects) were bothered by other people's oral malodour.

Perception of own oral malodour

Only 19.1 % of the subjects felt uneasy about their own oral malodour (Fig. 3).

The perception of own malodour was significantly related to the gingival symptoms and the discomfort in the oral cavity ($p < 0.001$). The gingival bleeding at brushing was found in 30.8% of those who were concemed with

their own oral malodour. While 37.9% reported malaise in the oral cavity when they got up, about half of them were never concerned with their own oral malodour. On the other hand, only 58.6% (267 subjects) of the subjects, who were concerned with their own oral malodour, found other people's oral malodour unpleasant.

Perception of other people's oral malodour

The number of subjects, who were conscious of other people's oral malodour, was 1019 (42.7%), which was more than twice the number of subjects who felt uneasy about their own malodour (Fig. 3). On the other hand, only 267 subjects (26.2%) of these 1019 subjects worried about their own malodour.

Behaviour when subjects were conscious of their own breath malodour.

When subjects were anxious about their own oral malodour, 68.4% chewed gum, 41.7% utilized toothpaste advertised specially for preventing bad breath, and only 15.1 % used mouthwash (Table 6). We also found avoidance behaviour in this group. Nine percent of them covered their noses with their hand, 12.3% keep a distance from others, while 7 subjects (1.5%) indicated that they do not like meeting other people. It has been suggested that such people might suffer from psychosomatic pathology, i.e. social phobia.

In those subjects, who were concerned with both their own and other people's oral malodour (n=267), their behaviour was also investigated. However, a significant difference was not observed between this group and the control group. In the group concerned with their own malodour but not with other people's malodour, no significant difference was found either. It was noted that 5 of 7 subjects, who did not like meeting other people, were included in this group. In fact, when a subject was concerned with only their own oral malodour rather than another person's oral malodour, this might be a feature of social phobia.

Table 6 : Behaviour when concerned about own oral malodour (multiple answers)

	Numbers		Bothered by other people's malodour		Not bothered	
Using chewing gum	312	68.4%	169	63.3%	83	60.1%
Using the anti-malodour dentifrice	190	41.7%	112	41.9%	49	35.5%
Using moutwash and spray	69	15.1%	34	12.7%	25	18.1%
Covering the mouth when facing another	42	9.2%	29	10.9%	9	6.5%
Keeping distance from another	56	12.3%	37	13.9%	13	9.4%
Shunning the public	7	1.5%	2	0.7%	5	3.6%
Cleaning the tongue	82	18.0%	44	16.5%	21	15.2%
Others	23	5.0%	12	4.5%	7	5.1%
TOTAL	456		267		138	

Behaviour when subjects were conscious of other people's oral malodour

On public transportation, 3.6% of the subjects, who were concerned with other people's oral malodour, covered their noses with their hand, but this number was increased to 6.5% in the 588 subjects who did not worry about own oral malodour. This ratio increased to 14.9% in the 229 subjects who took a preventive measure to control their own malodour (Fig. 4). On public transportation, 21.6% of the subjects averted their faces, but this ratio increased to 52.2 % in the subjects who were not concerned with their own oral malodour, and to 47.2% in the subjects who take a positive attitude to control their own malodour.

When facing another person, only 1.6% of the subjects covered their noses with their hand. This ratio increased to 3.1% in the 588 subjects who were not conscious of their own oral malodour, increased further to 5.2% in the 229 subjects who took a positive attitude to control their own malodour. When facing another person, only 4.0% of the subjects averted their faces, but the number increased to 9.9% in subjects who were not concerned with own malodour and to 7.4% in subjects who took a positive attitude to control their own malodour.

In all social situations, 22.9% keep their distance from oral malodour, but this ratio also increased to 53.1% in subjects who were not concerned with their

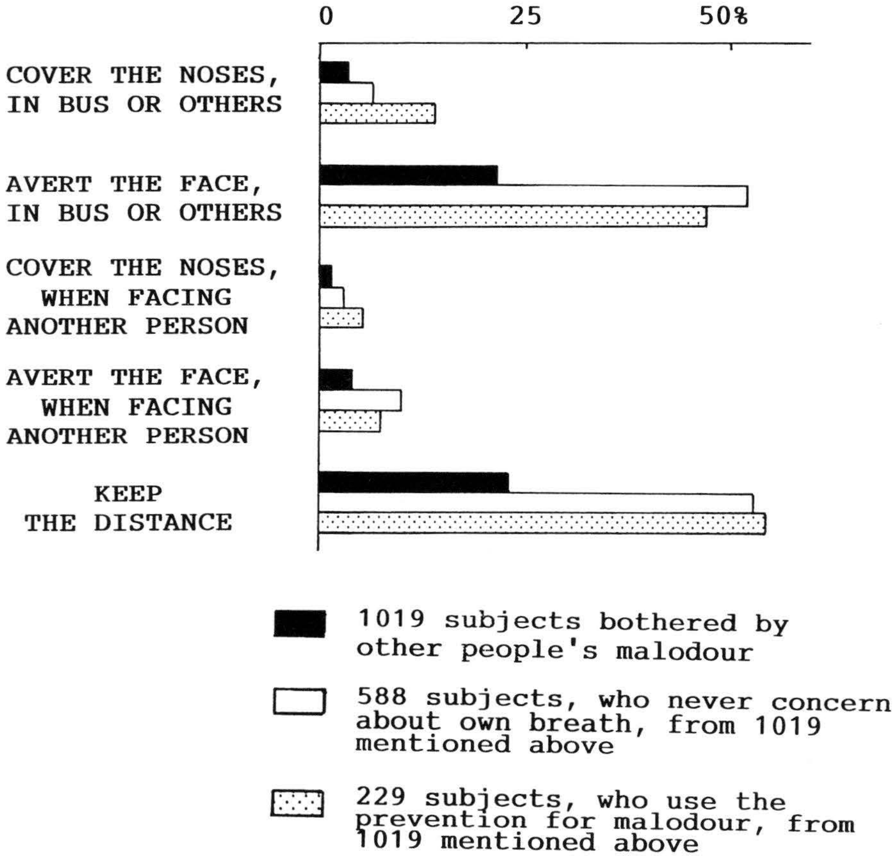


Fig. 4 : Of the subjects (n=1019) bothered by other people's oral malodour (black column), on public transportation, 3.6% covered their noses, and 21.6% averted their faces. But, when facing another person, 1.6% covered their noses, and 4.0% averted their faces. On the other hand, 22.9% kept their distance to avoid oral malodour. In the subjects (n=588) who were never concerned about their own oral malodour (white column), and in subjects (n=229) who used the preventive measures to control oral malodour (dotted column), the numbers dramatically increased.

own malodour and to 54.6% in subjects who took a positive attitude to control their own malodour.

Discussion

In Japan, psychosomatic halitosis has been named "Self-halitosis" in 1974, but this definition was dogmatic, and did not receive international recognition (2, 3). Rosenberg and co-workers (25) defined "Halitophobia", which was derived from the pathology of psychosomatic halitosis. A diagnosis of Halitophobia is applicable to most cases of psychosomatic halitosis. However, we have noticed that even ordinary halitosis patients have psychosomatic pathology rather than Halitophobia.

Since the incidence of halitosis was more than 50% (26), large numbers of patients were affected. However, only a few patients visited us because of their anxiety toward their own oral malodour.

A patient's strong anxiety about halitosis may be caused by the pathology of social phobia. We also speculate that the severe cases of social phobia with halitosis may be diagnosed as halitophobia. Namely, it has been shown that treatment or counseling for social phobia would be required in not only halitophobia but also ordinary halitosis patients.

Clinical observation indicated that patients are very afraid of other people's attitudes, behaviour and gestures (4, 5, 2). For example, the patients in our clinic believed that other people covered their noses with their hand because of the patient's oral malodour. They also believed that other people averted their faces from the patients because of the oral malodour. The patients also complained that when they entered into the office, their colleagues would open the window because of their halitosis. In severe cases, the patients could not face other people for fear of seeing their behaviour.

Japanese, especially women, often covered their mouth with the hand during speaking or facing someone because of the traditional habit that people did not like to show their white teeth while speaking. For example, Ohaguro, which stains teeth black to hide white teeth, was in use until about 100 years ago by married women and court nobility. People also believed that saliva or breath was extremely filthy. Thus, the guardians of Shinto shrines and Japanese buddhists wear paper masks during some religious services. Thereby, some Japanese may have unconsciously gotten into the habit of covering their mouth. However, patients believe that their behaviour was always caused by their oral malodour, because there was no evidence to prove that the behaviour was not caused by the oral malodour. Therefore, we surveyed people's perceptions and behaviours toward oral malodour to distinguish those halitosis patients who suffered social phobia.

The results indicated that only 456 subjects (19%) were concerned about their own oral malodour, but 1019 subjects (43%) were concerned with other

people's oral malodour. Since the incidence of oral malodour was expected to be approximately 50%, we found that about 60% of the subjects were never concerned with their own oral malodour, although they had oral malodour. As the majority of the subjects were not worried about own oral malodour, the patients who were extremely conscious about own oral malodour, were thought to suffer from some other pathology, such as social phobia, rather than halitosis.

Of the 878 subjects with gingival pathology who were advised that they had pathological oral malodour, only 138 subjects were bothered by their own oral malodour. Additionally, only 26% of the 1019 subjects, who were concemed with other people's oral malodour, were bothered by their own oral malodour. This implied that the subjects, who were concerned with their own oral malodour, focus on their own oral malodour without recognizing that others also have oral malodour. It has also been postulated that the most of the subjects, who were concerned with other people's oral malodour, were never concerned with their own oral malodour.

When subjects became concerned about their own oral malodour, 68%, 15% and 42% of subjects chewed gum, rinsed with mouthwashes, and used anti-malodour toothpaste, respectively. We found that a smaller number of subjects used mouthwash than we expected. There are several plausible reasons: 1) even the young generation of female Japanese might not have enough knowledge or education for oral health; 2) they may not believe the effectiveness of mouthwash on oral malodour; 3) some mouthwash may be unacceptable because of its strong taste; 4) mouthwash may be inconvenient to carry. Yaegaki *et al.* (1) have reported that subjects with a high knowledge of oral health do not believe the effectiveness of mouthwash. Therefore, a reliable and very effective mouthwash or other convenient procedure for preventing oral malodour should be developed.

Avoidance behaviours were also noted when the subjects worried about their own oral malodour. Nine per cent of subjects covered their noses with their hand, and 12% keep a distance from others, while 7 subjects indicated that they do not like meeting other people. We postulated that the avoidance behaviours, mentioned above, have been caused by psychological pathology, such as social phobia. Then the social relationships with other people may be obstructed by the avoidance behaviours, and the psychological social distance could be greater. If this is true, a vicious circle of social phobia might be repeated.

When the subjects are bothered by other people's oral malodour, 22% of the subjects on public transportation averted their faces from oral malodour, while only 4% covered their noses with their hand. Only 4% of the subjects, when

they encountered oral malodour when facing another person, averted their faces. We obtained a remarkable result that only 1.6% of the subjects, when facing another person, covered their noses with their hands. This fact indicates that people's behaviour of covering the noses is not caused by other people's oral malodour. Nevertheless every halitosis patient believes that their oral malodour causes this behaviour. The results provide good evidence to persuade patients to believe that their oral malodour does not make other people cover the noses.

However, the incidence of the behaviours dramatically increased in the subjects who were never concerned with their own oral malodour; 52.2% averted their faces on public transportation, and 6.5% covered their noses. When they were facing another person, 9.9% averted the faces and 3.1 % covered the noses. The results demonstrated that many halitosis patients, who reacted behaviourally toward other people's oral malodour, never concerned themselves with their own oral malodour.

It has been concluded that there are psychologically four groups in our subjects: 1) the people who were very concerned with their own oral malodour and frequently take avoidance behaviours toward it; 2) the people who chose simple behaviours, such as covering noses etc. to escape from other people's oral malodour, but were never concerned with their own oral malodour; 3) the people who took measures to prevent their own oral malodour, but sometimes take the simple behaviour, mentioned above, toward other people's oral malodour; 4) people who had no interest in oral malodour. We have considered that the results would be useful for the treatment of Halitophobia patients.

A study linking a person's behaviours toward oral malodour and a person's personality is in progress. If this relation is elucidated, we could give patients good suggestions how to perceive and how to cope with other people's behaviours such as covering their noses etc.

Acknowledgement

We are very grateful to Dr. Jeffrey M. Coil, Assistant Professor, Faculty of Dentistry UBC for his excellent assistance to prepare the manuscript. We also thank the staff of our laboratory, Department of Preventive Dentistry, Tsurumi University, for their supreme assistance.

References

1. Yaegaki K., Takano Y. and Suetaka T. (1989) Investigation of people's attitudes and reactions towards oral malodour. *Shigaku* 77:171-178. (in Japanese)
2. Yaegaki K. Sanada K. and Mirot F. (1993) Diagnostic et traitement de l'halitose. *J Parodont* 12:27-33.
3. Yaegaki K. (1995) Oral malodor and periodontal disease. In : Rosenberg M. (Ed), *Bad Breath: Research Perspectives*, Ramot Publishing, Tel Aviv University, Tel-Aviv, Israel, pp87-108.
4. Ozeki H., Amemiya H., Tanaka N., Kanamori K., Tojo H., Ushiyama T., Ishii Y., Narita R. and Uchida Y. (1991) Therapy of psychogenic halitosis and its problems. *Nihon, Shika-shinshin-igakkai zasshi* 6:76-80 (in Japanese).
5. Sugiura M. (1982) Approach to psychogenic halitosis. *Dental Outlook* 60:859-866 (in Japanese).
6. Tonzetich J. and Richter V.J. (1964) Evaluation of volatile odoriferous components of saliva. *Archs oral Biol* 9:39-45.
7. Tonzetich J. and Kestenbaum R.C. (1969) Odour production by human salivary fractions and plaque. *Archs oral Biol* 14:815-827.
8. Tonzetich J. (1971) Direct gas chromatographic analysis of sulphur compounds in mouth air in man. *Archs oral Biol* 16:587-597.
9. Tonzetich J. (1973) Oral malodour: an indicator of health status and oral cleanliness. *Int Dent J* 28:309-319.
10. Tonzetich J. and Johnson P.W. (1977) Chemical analysis of thiol, disulfide and total sulphur content of human saliva. *Archs oral Biol* 22:125 -131.
11. Tonzetich J. and Johnson P.W. (1986) Radiochromatographic analysis of H_2S-collagen complex in rat-tail type I collagen. *Archs oral Biol* 82:297-300.
12. Preti G., Clark L., Cowart B.J., Feldman R.S., Lowrt L.D., Weber E. and Young I.M. (1992) Non-oral etiologies of oral malodor and altered chemosensation. *J Periodontol* 63:790-796.
13. Johnson P.W., Yaegaki K. and Tonzetich J. (1992) Effect of volatile thiol compounds on protein metabolism by human gingival fibroblasts. *J Periodont Res* 27-553-561.
14. Ng W. and Tonzetich J. (1984) Effect of hydrogen sulfide and methyl mercaptan on the permeability of oral mucosa. *J Dent Res* 63:994-997.
15. Kaizu T. (1976) Analysis of volatile sulphur compounds in mouth air by gas chromatography. *J Jpn Assoc Periodontol* 18:1-12 (in Japanese).
16. Yaegaki K. and Suetaka T. (1989) The effect of mouthwash on oral

malodour production. *Shigaku* 76:1492-1500 (in Japanese).
17. Yaegaki K. and Suetaka T. (1989) Fractionation of the salivary cellular elements by percoll density gradient centrifugation and the distribution of oral malodour precursors. *Shigaku* 77:269-275 (in Japanese).
18. Yaegaki K. and Suetaka T. (1989). The effect of zinc chloride mouthwash on the production of oral malodour, the degradations of salivary cellular elements and proteins. *J Dent Hlth* 39:377-386.
19. Yaegaki K. and Suetaka T. (1989) Periodontal disease and precursors of oral malodorous components. *J Dent Hlth* 39:733-741.
20. Yaegaki K. and Sanada K. (1992) Effects of a two-phase oil-water mouthwash on halitosis. *Clin Prev Dent* 14:5-9.
21. Yaegaki K. and Sanada K. (1992) Volatile sulfur compounds in mouth air from clinically healthy subjects and patients with periodontitis. *J Periodont Res* 27:233-238.
22. Yaegaki K. and Sanada K. (1992) Biochemical and clinical factors influencing oral malodour in periodontal patients. *J Periodontol* 63:783-789.
23. Yaegaki K, Tonzetich J, Johnson PW (1996). Effect of methyl mercaptan on synthesis and degradation of collagen in human gingival fibroblasts. *J Periodont Res* (in press).
24. Yaegaki K., Masui I., Sano S. and Kitamura T. (1995) Studies for behavior and perception toward oral malodour. *Tsurumi-shigaku* 21:457-466 (in Japanese).
25. Rosenberg M. and Leib E. (1995) Experiences of an Israeli Malodor Clinic. In: Rosenberg M. (Ed) *Bad Breath: Research Perspectives*, Ramot Publishing, Tel Aviv University, Tel-Aviv, Israel, pp 137-148.
26. Sulser GF, Brening RH, Fosdick LS (I 939). Some conditions that effect the odor concentration of breath. *J Dent Res* 18:355-359.

The Olfactory Reference Syndrome and Halitosis

*B.E. Johnson**

Dentists and primary care physicians are the health professionals who are most commonly confronted with the clinical problem of halitosis. The condition may be identified by the medical doctor or dentist, or brought as a complaint by the patient. The patient who comes with this complaint frequently has had the malodour identified by a spouse, family member, or friend.

Occasionally, a patient presents complaining of halitosis, but at no time does the medical doctor or dentist detect a foul odour. The patient may be greatly distraught by the presumed malodour, stating that bad breath is separating him or her from friends, is interfering with a job, or is causing avoidance of social interaction. Such a person may be experiencing a syndrome called the olfactory reference syndrome. This syndrome might best be introduced by a case report.

Case 1. GN - A 36 year old female

This woman consulted the physician for the complaint of halitosis. GN recently had become "aware" of bad breath. She believed that people readily noticed the odour and distanced themselves from her. This was most evident, according to her perception, at sales presentations. Not long before she had noticed the odour, she had received a promotion to a direct sales position from a telemarketing job.

GN had no current illnesses or physical complaints. She had had no surgeries or hospitalizations. She had normal menstrual cycles and had never been pregnant. She did not take medications or vitamin supplements. GN did not smoke cigarettes and, while she drank alcohol occasionally, she never drank during the workday. She ate meals three times daily, only infrequently skipping lunch.

The patient was unmarried. Her job promotion had resulted in a recent move to Kansas City and she had no roommate or close friend.

The general physical examination was normal; at no time during the visits was an oral malodour noticed. Blood and urine tests, along with radiographs

---

* B.E. Johnson, Department of Medicine, University of Kansas Medical Center, 3901 Rainbow Blvd., Kansas City, KS 66160, USA.

of the sinuses and chest, were normal. An esophagogram and upper gastrointestinal series were also normal.

At each subsequent visit, GN was more distraught. She was convinced that her odour was interfering with her success in sales work. She refused to believe that no odour was noticed, and no disease was detected. She refused a trial of antidepressants or a neuroleptic agent. She left the practice and has not returned.

There are a series of syndromes which are classified by psychiatrists as monosymptomatic hypochondriachal psychoses.

Among the groupings of the monosymptomatic hypochondriachal psychoses are the syndromes categorized by the following three delusions :

* delusion of being infested with parasites
* delusion of being physically misshapen
* delusion of emitting offensive body odour.

The delusion of emitting offensive body odour has acquired multiple synonyms over the years (Table 1). These names reflect the changes in thinking regarding this syndrome. Earlier characterization of these disorders suggested that the patient was psychotic (group "a").

Subsequent investigation led to the description of these disorders as either hallucinations (group "b") or phobias (group "c"). Current thinking, not completely accepted by all psychiatrists, describes the syndrome in terms that encompass several different etiologies.

Table 1 : Synonyms for the false perception of a body odour

a.	Monosymptomatic hypochondriacal psychosis Monosymptomatic psychosis Solitary psychosis
b.	Chronic tactile hallucinosis Tactile delusional hallucinosis
c.	Hypochondriacal paraphrenia Bromidrosiphobia Autodysosmophobia
d.	Olfactory delusional syndrome Olfactory reference syndrome

The term often used to define a delusion of emitting offensive body odour is the "olfactory reference syndrome". This syndrome is variously described as "a delusion (involving) bodily functions or sensations" (1) and as "a single

hypochondriacal, delusional system, distinct from the remainder of the personality ... (the) delusion may be accompanied by illusional misperceptions or, at times, by poorly defined hallucinations." (2). The caveat in these definitions is that, while the patient is deluded or obsessed with an imagined odour, the remainder of the personality is relatively intact.

The term "olfactory reference syndrome" was first coined by Pryse-Phillips in 1971 (3). He described patients, like GN in case 1, who have a preoccupation with imagined body odours. These patients believe that an internal source of odour, be it sweat, intestinal gas or breath, is eminating from their bodies. These patients also feel that others can readily detect the odour. The belief that the odour offends other people is deeply distressing to the patients. Pryse-Phillips noted that the age of onset of this disorder is relatively young, averaging about 25 years. The majority of patients are male (78%). A large percentage (35%) do not work, although his sample was drawn mostly from mental hospitals and psychiatric practices. It is acknowledged that most patients with the olfactory reference syndrome never present to psychiatrists or psychiatric wards because the disorder, although interfering with their lives, uncommonly results in their incapacitation (4,5).

The olfactory reference syndrome typically is present for some time, often years, before recognition. The patients may have sought medical help on numerous occasions, usually unsuccessfully, because of both an actual absence of bad breath and poor recognition of the olfactory reference syndrome by professionals. "Unfortunately, many physicians, especially ENT specialists, are unaware of this condition and many patients are over-investigated and even surgically treated to no avail." (4). The onset is most often insidious, although a third of affected patients may describe a sudden, precipitating event. Precipitating events include sino/pulmonary infection, comments by family or friends regarding halitosis, job change, personal loss or any of a wide range of other factors. The majority of patients have an antecedent psychiatric history, though usually for personality disorders, not psychotic illness.

Patients with the olfactory reference syndrome often have a pre-morbid personality characterized by obsessional, perfectionist traits (6). Patients may also have been insecure or sensitive persons, greatly influenced by the comments or actions of others. The disorder is considered a discrete maladaptation, with maintenance of what otherwise is a functional personality. However, when the syndrome fully develops the effect on the patient's life can be dramatic and the disruption profound. Patients adopt compulsive and obsessive activities. These complicated activities are counterphobic rituals which reflect a "contrite reaction" (7). Patients withdraw

from social situations. Frequent toothbrushing, cleansing and use of mouthwash are common. These obsessive traits reflect the lengths to which patients will go to prevent their perceived malodour from affecting others. One patient even believed that other drivers on the streets kept their windows rolled up to avoid his smell (4).

Patients with this syndrome are extremely sensitive to minor facial grimaces, body movements or innocent comments by others. Typical movements of persons during normal conversation are misinterpreted and magnified by the patient. This misinterpretation reinforces the belief that others are offended by the patient's breath. Rumination on these false beliefs results in heightened anxiety and embarrassment. Patients become "quietly paranoid," isolating themselves from social contact and, if untreated, become almost reclusive (5).

Patients with the olfactory reference syndrome may either fail in selected endeavors, excuse past failure, or fear future failure as a consequence of the perceived oral malodour. Their delusions of halitosis become an excuse for poor work performance, loss of friends, separation from family, or lack of confidence in social settings. In many cases, this fear of failure becomes self-fulfilling (8). The woman in case 1, GN, was fearful of not succeeding in her new position, which required much closer social interaction than her previous telemarketing job. It is not difficult to imagine her attributing a substandard job performance to the perceived halitosis.

A second case may illustrate additional characteristics of the olfactory reference syndrome.

Case 2. VA - A 29 year old male

This man consulted the physician with complaints of excessive perspiration and halitosis. He was a trainee for the ministry and needed to complete his current year successfully to be considered for ordination and placement in his own parish. He had become increasingly self-conscious around congregants because of a perception of his own excessive perspiration and halitosis. Even during the winter, VA thought he perspired excessively. He took up to four showers a day and used a bottle of mouthwash (16 ounces or 450 ml) every three to four days.

He was convinced that his congregants were repelled by his odours. He described in great detail how innocent gestures "really" meant people were "disgusted" with his smell. Normal group movement was interpreted as a deliberate attempt to avoid him. VA was certain that poor performance marks

from the congregation would prevent his advancement.

He had no current illnesses or physical complaints. He did not take any medications except for several vitamin preparations daily. His only surgery had been an appendectomy. VA admitted to several previous medical workups for his complaints.

The patient was unmarried with no roommate or close friends. He did not smoke or drink alcohol. He had no atypical dietary habits. He was only temporarily in Kansas City for this training internship.

Physical examination performed at several office visits was normal with no halitosis and no excess perspiration noted. Blood and urine tests, thyroid studies, urine for catecholamines, chest x-ray and even a 24-hour monitor for blood pressure were all normal.

A trial of medication was started for the exessive perspiration; this trial eventually included aluminium chloride (hexahydrate), propantheline, and clonidine (both oral and patch). The patient complained bitterly of poor response to these interventions, and that the problem continued unabated.

Eventually VA was transferred out of Kansas City. A chance conversation at a later time with several congregation members confirmed that his obsessive characteristics had been noticed and that, in fact, a mediocre evaluation had been forwarded to church headquarters.

The two cases, GN and VA, share several salient features. Both patients had jobs in which there was a new requirement to work closely with people who would determine the patient's success. Both perceived their disorders with no "independent" confirmation; neither patient was married, had a roommate, close friend, or family member living with them. They felt they could trust no one to give a correct response as to whether the offending odour existed. Both patients had developed complex rituals designed to hide or deflect the perceived odour from others. GN stood close to her displays rather than more directly encountering clients; she frequently refused dining invitations, and she rarely offered clients a ride in her car. VA showered and gargled obsessively. Both patients refused to believe that no organic diagnosis was forthcoming, and both failed any attempted intervention. Both left the practice before psychiatric assistance could begin.

Patients with the olfactory reference syndrome may have elements of several conditions, including symptoms of depression, neurosis, or somatization to accompany their obsessive-compulsive thoughts and rituals. Although the patients may otherwise be quite rational, a profound illogicality exists regarding the delusion. The patient places strong demands on the physician or dentist to intervene but often refuses to consider psychiatric treatment.

Because of the many manifestations of the syndrome, a single treatment regimen has not proven to be uniformly successful. Psychotherapy has been attempted and may be useful for patients who have neurotic components to their personality. Obsessive-compulsive patients are particularly difficult to treat. Patients with identifiable depressive symptoms have responded to the use of anti-depressants (4, 5, 9). Tricyclic antidepressants have been most widely reported for this latter symptom; use of the newer serotonin uptake inhibitors has not been described.

Many reports advocate the use of the neuroleptic pimozide (2, 5, 7, 8, 9). Pimozide is an inhibitor of dopamine uptake in the central nervous system. In the United States, pimozide officially carries an indication for treatment of complex tics such as those found in the Tourette syndrome. When used in patients with the olfactory reference syndrome, pimozide interrupts the delusional thinking and provides some degree of symptom relief in a few days. Successful amelioration of the syndrome requires 6-8 weeks of therapy. For those who do respond, pimozide appears to be required as maintenance therapy for an indefinite length of time. Early discontinuation of the medication results in the return of delusions and re-adoption of avoidance activities. Three-quarter of the patients treated with pimozide respond; the remainder of patients are suspected of having a second, underlying disorder requiring a different treatment.

Even though the olfactory reference syndrome is described almost exclusively by psychiatrists, it should be more widely appreciated by other health deliverers. Dentists, periodontologists, primary care physicians, and otolaryngologists are occasionally consulted by patients with the complaint of halitosis. Most true oral malodour is caused by an identifiable, treatable condition. But not all cases will have an organic cause, and early recognition of the olfactory reference syndrome is a far more appropriate response than extensive investigation or surgery.

References

1. American Psychiatric Association (1994) Diagnostic and Statistical Manual of Mental Disorders, fourth edition. Washington, D.C., American Psychiatric Association, pp 298.
2. Munro A. and Chmara J. (1982) Monosymptomatic hypochondriacal psychosis : A diagnostic checklist based on 50 cases of the disorder. *Can J Psychiatry* 27:374-376.
3. Pryse-Phillips W. (1971) An olfactory reference syndrome. *Acta Psych*

Scand 47:484-509.

4. Malasi T.H., El-Hilu S.R., Mirza I.A. and El-Islam M.F. (1990) Olfactory delusional syndrome with various aetiologies. *Br J Psych* 156:256-260.
5. Goldberg R.L., Buongiorno P.A. and Henkin R.I. (1985) Delusions of halitosis. *Psychosomatics* 26:325-331.
6. Videbech T. (1966) Chronic olfactory paranoid syndromes. *Acta Psych Scand* 42:183-213.
7. Bishop E.R. (1980) Monosymptomatic hypochondriasis. *Psychosomatics* 21:731-747.
8. Munro A. (1980) Monosymptomatic hypochondriacal psychosis. *Br J Hosp Med* 24:34-38.
9. Brotman A.W. and Jenike M.A. (1984) Monosymptomatic hypochondriasis treated with tricyclic antidepressants. *Am J Psych* 141:1608-1609.

Treatment of Breath Odour

The efficacy of a combined zinc and triclosan system in the prevention of oral malodour

*S.J. Raven, J.R. Matheson, E. Huntington and J. Tonzetich**

Introduction

The incidence of oral malodour in the general population is high, with many people experiencing problems such as "morning mouth". Mass marketed dentifrice and mouthwash can play an important role in helping to reduce both the incidence and the extent of oral malodour. In this paper the requirements of a mass market product are discussed and data presented to demonstrate the efficacy of products containing the combination of zinc and triclosan. The efficacy is demonstrated using a variety of evaluation techniques and the importance of product form and frequency of use are discussed.

For a product to be effective and suitable for mass marketing it must satisfy a number of criteria. It should obviously be safe for unsupervised use, have acceptable sensory properties to encourage regular use and be convenient as well as cost effective. In addition, it should contain an active species such as an anti-microbial agent, a compound capable of complexing volatile odour components, or an odour masking ingredient. The presence of an active species does not necessarily guarantee clinical efficacy and thus product efficacy should also be demonstrated in a clinical study against a suitable control.

Selection of an active system

One of the most studied active ingredients for the prevention of oral malodour is zinc. It was first examined in the 1970s when it was shown to give significant reductions in volatile sulphur compounds, a key component of oral malodour (1). A number of routes have been postulated for the role of zinc in reducing volatile sulphur compounds (VSC). It may complex VSC to form insoluble non-odorous zinc sulphides (2) or reduce the level of oral

---

* S.J. Raven[1], J.R. Matheson[1], E. Huntington[1] and J. Tonzetich[2], 1 Unilever Research Port Sunlight Laboratory, Quarry Road East, Bebington, Wirral L 63 3JW, United Kingdom
2 Department of Oral Biology, University of British Columbia, Vancouver, BC, Canada V6T 1Z3.

microorganisms (3) through its antimicrobial action. More recently zinc has been shown to reduce the degradation of salivary cellular elements via the inhibition of thiol proteinase activity (4).

In addition to zinc, other antimicrobial agents such as cetylpyridium chloride (CPC) and triclosan (2,4,4-trichloro-2-hydrodiphenylether) have also been used to reduce oral malodour. Triclosan (TCN) is an ideal agent for this purpose due to its broad antimicrobial activity and compatibility with other compounds typically used in oral care formulations. The combination of zinc and triclosan has also been shown to be a particularly effective system in the control of plaque and gingivitis (5).

Delivery of the active ingredient

The overall efficacy of the product will be influenced by the ability of the active ingredient to be delivered and retained in the oral environment (6). The importance of substantivity is illustrated by the second agent zinc, which although a relatively mild antibacterial agent has clinical anti-plaque activity because of its high substantivity to buccal surfaces. The delivery and retention of zinc and triclosan in the oral cavity can be examined using oral pharmacokinetic studies which measure the concentration of the active ingredients in saliva following product use. Typical half lives for zinc and triclosan in the oral environment when delivered by a toothpaste are 50.4 min^{-1} and 23.6 min^{-1} respectively (7).

The delivery of one of the agents, TCN, discovered in this paper is particularly sensitive to the formulation and it has been shown that product structure can have a direct impact on its efficacy as an antiplaque agent (8)

Evaluation of product efficacy in the prevention of oral malodour

There exists a wide range of techniques for the evaluation of agents in the prevention of oral malodour, ranging from simple *in vitro* tests to full clinical studies. To gain a realistic evaluation of the efficacy of a fully formulated product it is essential that *in vivo* techniques are utilised. A number of different options exist to measure product efficacy, and these are illustrated in Fig. 1 together with their relationship to the mechanism of odour generation.

One of the potential pathways to malodour is the breakdown of sulphur containing amino acids to form volatile sulphur compounds. The effect of products on this pathway can be assessed by examining changes either in

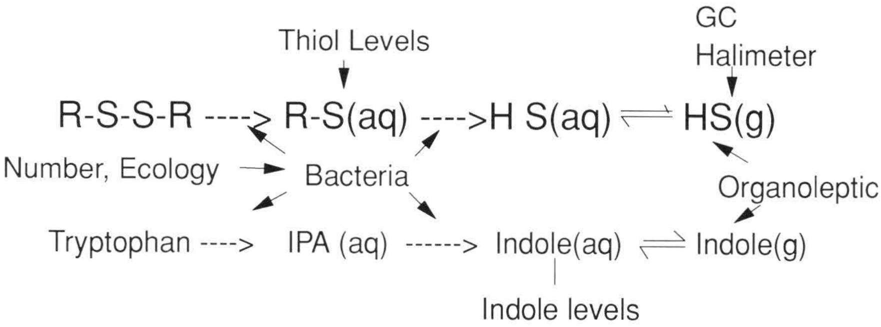


Fig. 1 : Routes for evaluation of product efficacy.

the concentration of thiol intermediates present in saliva or in the level of volatile sulphur compounds in mouth air. Similar analysis can be carried out for other proposed pathways such as the generation of indole from tryptophan. However, often the detection levels of the final volatile gas is below current instrumental detection limits and the analysis of the intermediates is the only route available. The overall impact of the product on oral malodour can be assessed using clinical studies involving trained odour assessors or alternatively by monitoring the effect on the agent of bacterial numbers *in vivo*.

Measurement of thiol levels in saliva

Protocols have been developed for the measurement of thiol levels in salivary samples and the influence of products on these levels (9). A typical method involves collection of a salivary samples from panellists prior to product use and at set time intervals after brushing/rinsing. Saliva samples are incubated for 24 hours and then analysed for thiol using a colorimetric method utilising the reagent 4,4'-bis(dimethylamino)diphenyl carbinol. The result obtained following the use of a dentifrice containing zinc (0.23%) is given in Fig. 2. The zinc containing dentifrice showed a significant benefit compared to the placebo paste (10).

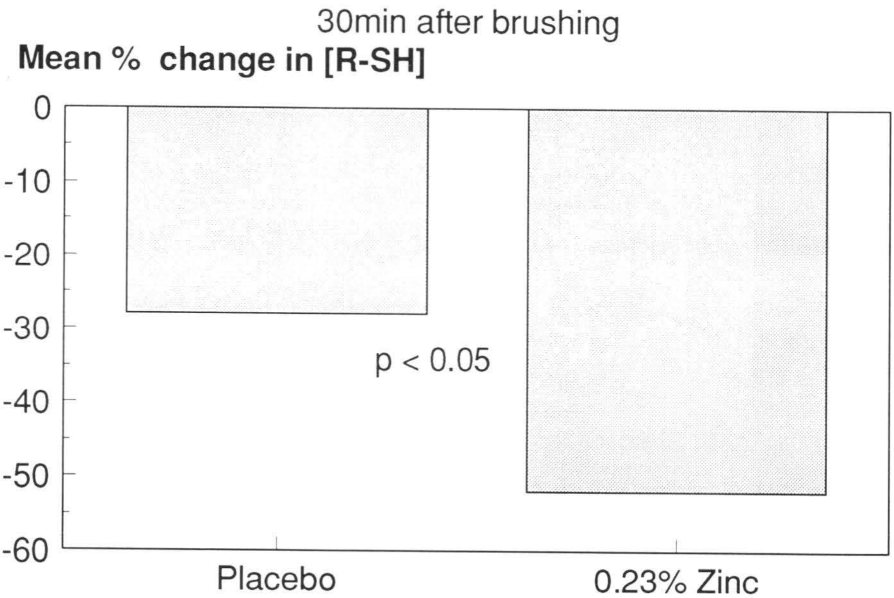


Fig. 2 : Reduction in salivary thiol levels following use of a zinc dentifrice.

Analysis of mouth air

One of the most relevant methods for the assessment of oral malodour is the direct analysis of mouth air. An estimate of the odour intensity can be gained from analysis of the expelled air of panellists using trained odour judges. The principal limitation of this method is the subjective nature of the human nose and therefore care should be taken to have sufficient panel size and adequately trained odour judges.

An alternative method is to use the instrumental analysis of air although this is often limited by the concentration of the odour components being below detection levels. Instrumental techniques have been most successfully employed for analysis of volatile sulphur compounds where traditionally gas chromatography has been employed (11). More recently the Halimeter (Interscan Corp., Chatsworth, CA, USA) has been used by a number of workers (12, 13).

An example of the data from an organoleptic study is shown in Fig. 3. In this crossover study panellists used 10 ml of a water placebo, commercial mouthwash (Listerine©) or an experimental mouthwash containing 0.15%

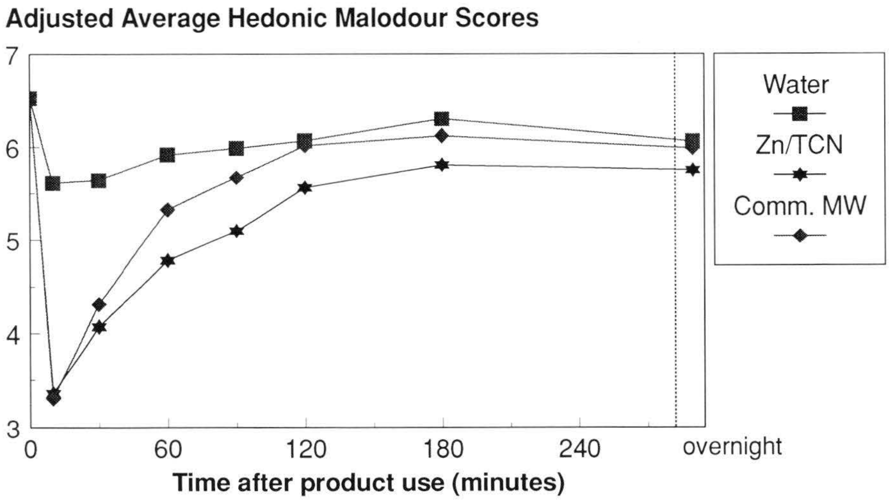


Fig. 3 : Results of hedonic study using a Zn/TCN mouthwash.

triclosan and 0.84% zinc (as zinc sulphate heptahydrate). A panel of odour judges evaluated the mouth air of panellists prior to product use and at 5, 30, 60, 120, and 180 minutes after rinsing. Mouth air was also evaluated in the morning following product use the previous evening. The experimental Zn/TCN mouthwash can be seen to give a large immediate benefit relative to the placebo with reductions in odour still significant over placebo even after the overnight period ($p < 0.05$). The product containing zinc and triclosan was observed to give a more prolonged reduction in odour than the commercial mouthwash tested.

The influence of product type and habit on efficacy

The overall benefit obtained from a commercial oral care product can also be influenced by the product type. In addition, individual habits may play a role as these affect both the frequency and method of use, for example the amount of product applied and whether subsequent rinsing takes place.

Malodour efficacy of toothpaste versus mouthwash

The relative efficacy of toothpaste and mouthwash containing zinc and triclosan, was evaluated by the analysis of mouth air using a gas chromatograph fitted with a sulphur specific flame photometric detector. The percent reduction in total VSC was calculated by comparison of the sum of the H_2S and CH_3SH levels relative to the baseline score prior to product use. Analysis was undertaken using two-way ANCOVA with baseline scores as covariate. Each study involved two products, a test and a control with the zinc and triclosan active agents omitted. In the toothpaste studies panellists brushed for one minute with 1.5 g of either test toothpaste (0.3% TCN, 0.23% $zinc^{2+}$ as zinc citrate trihydrate) or placebo, followed by rinsing with 10 ml water for 30 seconds. In the study using mouthwash panellists rinsed for one minute with 10 ml of either active (containing 0.15% TCN, 0.84% $zinc^{2+}$) or placebo product. Two different time periods were used in the studies. In the shorter term studies the mouth-air was analysed over a three hour period immediately following product use. In the extended use studies mouth air samples were taken in the early morning prior to any consumption of drink, food or use of oral care products on day 0 (baseline), 1, 7, 14, and 21. All studies were of a crossover design and employed male nonsmokers. In the long term studies a three week interval was introduced between the two phases to prevent any carry over effects. In total three separate studies were carried out. The protocols are summarised in Table 1 and results given in Tables 2, 3, 4 and 5.

Table 1 : Protocols of in vivo VSC studies

Study	No of Subjects	Test product	Product type	Period measured
1	16	0.3% TCN, 0.23% Zn	Toothpaste	0, 1, 10, 60, 120 and 180 min
2	24	0.3% TCN, 0.23% Zn	Toothpaste	0, 1, 7 and 21 days
3	17*	0.15% TCN, 0.84% Zn	Mouthwash	0, 1, 10, 60, 120, 180 min and 1, 7, 14 and 21 days

* Only 10 randomly selected subjects were analysed in the 0-3 hour period.

Table 2 : Results of Short Term Toothpaste Study

Time (min)	Total VSC (ng VSC/10 ml mouth air)		Statistical Significance
	Zn/TCN mean (SE)	Placebo mean (SE)	p ≤
0	12.54 (1.59)	10.91 (1.34)	-
1	2.46 (0.36)	11.41 (1.48)	0.0001
10	3.22 (0.46)	13.80 (1.94)	0.0001
60	6.72 (1.04)	12.50 (1.52)	0.0001
120	6.98 (1.45)	11.50 (1.31)	0.0003
180	9.73 (1.33)	13.06 (1.54)	0.023

Table 3 : Results of long term toothpaste study

Time (days)	Total VSC (ng VSC/10 ml mouth air)		Statistical Significance
	Zn/TCN mean (SE)	Placebo mean (SE)	p ≤
0	8.12 (1.21)	7.91 (0.16)	-
1	6.91 (1.24)	8.19 (1.48)	0.116
7	6.36 (1.16)	8.00 (1.17)	0.087
21	5.15 (0.86)	8.32 (1.37)	0.006

Table 4 : Results from short term mouthwash study

Time (min)	Total VSC (ng VSC 10/ml mouth air)		Statistical Significance
	Zn/TCN mean (SE)	Placebo mean (SE)	p ≤
0	11.04 (1.80)	8.26 (1.12)	-
1	1.34 (0.24)	6.32 (1.92)	0.010
10	1.01 (0.17)	5.85 (1.52)	0.0193
60	0.88 (0.26)	4.48 (1.41)	0.0125
120	1.57 (0.60)	6.19 (2.20)	0.0420
180	1.73 (0.57)	5.86 (1.44)	0.0038

Table 5 : Results from long term mouthwash study

Time (days)	Total VSC (ng VSC/10 ml mout air)		Statistical Significance
	Zn/TCN mean (SE)	Placebo mean (SE)	p ≤
0	10.75 (1.35)	10.65 (1.71)	-
1	3.62 (1.07)	5.20 (0.92)	0.0330
7	3.41 (1.01)	7.45 (1.75)	0.0043
14	2.30 (0.79)	7.78 (1.63)	0.0097
21	1.86 (0.49)	5.60 (1.01)	0.0028

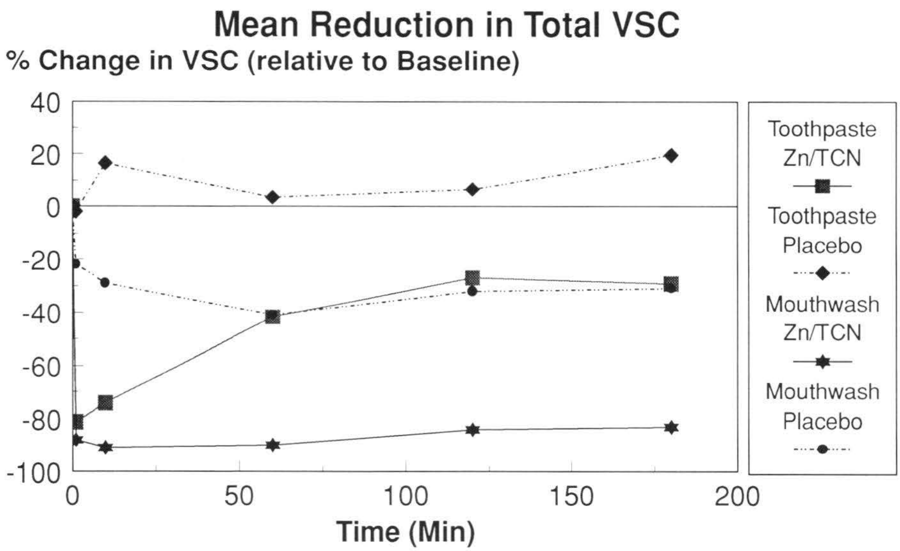


Fig. 4 : Toothpaste vs mouthwash

Short term efficacy

The combined results of the short term studies on both mouthwash and toothpaste are shown in Fig. 4. Total VSC levels are given for active and control products over the 3 hour testing period. In both cases the active product, containing zinc and triclosan, gave a significant benefit ($p < 0.05$) over the respective control. Toothpaste gave a similar benefit to mouthwash immediately after use. However the mouthwash was seen to maintain its

effect for a longer period of time with reductions in VSC's of greater than 80% still maintained after 3 hours. A benefit was also seen over baseline for the placebo mouthwash.

Influence of regular product usage on efficacy

The results of regular product use are illustrated in Fig. 5. This shows the mean total VSC level for each product over the study period. For toothpaste the test product showed a significant benefit ($p < 0.01$) over the placebo product after 21 days. In addition the effect of the toothpaste with respect to the control was seen to increase significantly over the three week test period ($p< 0.05$) when analysed for a linear trend (t-test). Similar results were seen with the mouthwash, with the test product showing a significant benefit over the placebo at all time points and a significant increase with respect to the placebo over the three week period ($p< 0.05$).

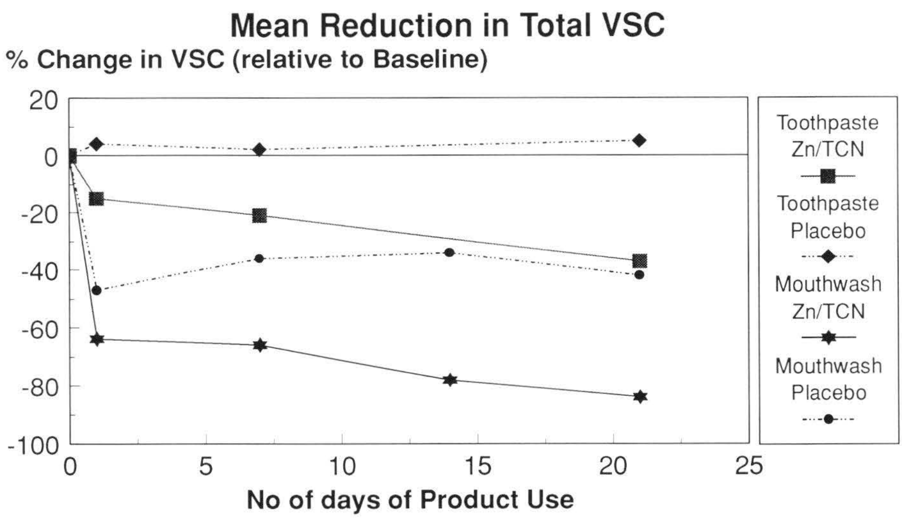


Fig. 5 : Extended product use.

Discussion

Efficacy of systems containing zinc and triclosan

The data clearly demonstrate a significant benefit in reductions of the volatile sulphur compounds can be obtained by addition of the active ingredients, zinc and triclosan to either toothpaste or mouthwash. These results are in agreement with previous work in which both zinc and triclosan individually have been shown to give significant reductions in oral malodour (14-16).

Highest reductions in malodour are seen immediately after product use, with mouthwash and toothpaste giving reductions compared to the baseline of 88% and 80% respectively. This time corresponds to the period when the active agents are in the highest concentration. With toothpaste the levels of VSC were seen to increase slowly over the three hour period whereas with the mouthwash the efficacy of the product was maintained. Both the mouthwash and toothpaste demonstrated efficacy over long time periods with significant reductions in oral malodour observed following an overnight period. This extended effect could be due to zinc, which has been shown to have good oral substantivity (7).

Effect of long term product usage

When the products were used regularly over a three week period, a steady decrease in the baseline VSC levels of the subjects was observed. This decrease was most pronounced with the toothpaste where the decrease in VSC levels, relative to the baseline, went from 15% at day 1 to 37% on day 21. With mouthwash the reduction in VSC levels from 66% to 83% over the same period.

A number of reasons for this increased efficacy can be postulated. One possibility is that levels of the active ingredients in saliva never fall to their original values between treatments and thus gradually increase over the period evaluated until they reach an elevated steady state. A second, perhaps more plausible, reason is that the number of bacteria in the oral cavity is decreased due to the antimicrobial action of the zinc/triclosan combination and hence the odorigenic potential of the microflora is reduced. Evidence to support this hypothesis comes from the plaque and gingivitis studies previously performed on these product which demonstrated that plaque levels in subjects were reduced and gingival health improved (5). The improvement

in gingival health itself could lead to a reduction in odour as periodontal pockets are believed to be an important source of oral malodour (17). A third possibility is that the ecology of the oral microbiota is shifted to a less odorigenic population. Evidence from oral ecology studies on subjects using zinc and triclosan for 7 months however showed no shift in microbiota and thus this possibility is unlikely (18).

Efficacy of a mouthwash compared with a toothpaste

If the toothpaste and mouthwash are compared the mouthwash gives slightly greater reductions in malodour initially (88% versus 80% respectively). This difference however increases with time and the respective reductions for mouthwash and toothpaste at three hour are 84% versus 22% and overnight 66% versus 15%.

A number of reasons may explain the higher efficacy of the mouthwash. With mouthwash a higher dosage of the active agents is given with each product use. The amounts of active delivered from a 10 ml dose of the mouthwash are 15 mg of zinc and 8.4 mg of triclosan. In comparison the 1.5 g dose of toothpaste delivers only 4.5 mg of zinc and 3.5 mg of triclosan.

A second potential benefit of the mouthwash is that by the rinsing action the product is distributed throughout the oral cavity whereas toothpaste is only applied to the teeth. The oral soft tissues are thought to act as a reservoir to help retain active agents and thus the greater coverage by the mouthwash may lead to increased retention and consequently explain the ability of the mouthwash to reduce odour over a longer period. The ability of the product to reach the tongue is especially important given the role of the tongue microflora as a key source of malodour.

A third possibility for the higher efficacy of the mouthwash is that in the protocol covering toothpaste use the panellists were asked to rinse with 10 ml of water after brushing. A consequence of this rinse is that the levels of active agents in the oral cavity will be reduced. Data to support this hypothesis comes from work undertaken on fluoride delivery. In one study (19) the fluoride delivered from a mouthwash was compared with that delivered from mouthwash followed by rinsing, toothpaste use followed by rinsing and rinsing with a toothpaste slurry. All samples were formulated to give the same total fluoride level. The results are summarised in Fig. 6. It can be seen that the salivary fluoride levels after toothpaste use are comparable with the mouthwash when both are following a rinse. In addition it demonstrates that use of a toothpaste slurry will give similar delivery to a mouthwash.

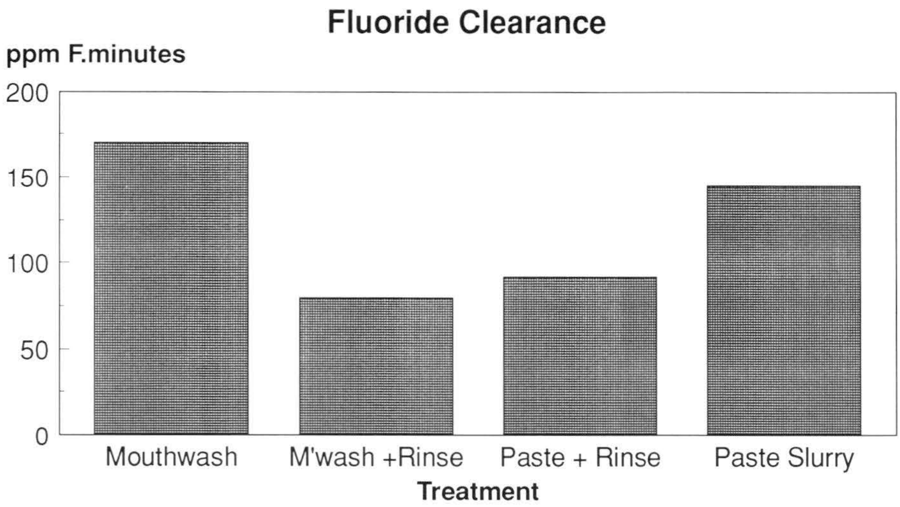


Fig. 6 : Delivery toothpaste vs mouthwash

The greater efficacy observed with mouthwash is likely to be because of a combination of the above factors. However it is clear that the effect of toothpaste may be increased by refraining from rinsing after brushing and possibly spilling toothpaste slurry around the oral cavity. In addition, regular use of the dentifrice will also result in an increased level of efficacy . This is important because the majority of the population of the western world uses toothpaste daily whereas the use of mouthwashes is far less common and irregular.

Summary

1. The combination of zinc and triclosan has been demonstrated to be effective in the reduction of oral malodour. The efficacy was measured using studies involving assessment of the reductions in volatile sulphur compounds in mouth air, thiol levels in saliva and mouth odour levels.

2. Regular use of products containing the dual active system increased the efficacy of both the dentifrice and mouthwash. The increase in efficacy is especially noticeable for the dentifrice.

3. Although after a single use mouthwash has higher efficacy than a dentifrice, the benefit of the dentifrice could potentially be increased by stopping postbrush rinsing, swilling the toothpaste around the oral cavity and regular

use of the product. The last point is important because mouthwash is often used on an irregular base whereas dentifrice is commonly used on a daily basis.

References

1. Tonzetich J. (1977) Production and origin of oral malodour : a review of mechanisms and methods of analysis. *J Periodontol* 48:13-20.
2. Boulware R.T., Southard G.L. and Yankell S.L. (1985) Sanguinaria extract, a new agent for the control of volatile sulfur compounds in the oral cavity. *J Soc Cosmet Chem* 36:297-302.
3. Kleinberg I. and Westbay G. (1992) Salivary and metabolic factors involved in odor formation. *J Periodontol* 63:768-775.
4. Yaegaki K. and Suetaka T. (1989) The effect of zinc chloride mouthwash on the production of oral malodour, the degradations of salivary cellular elements and proteins. *J Dent Hlth* 39:378-386.
5. Svatun B., Saxton C.A. and Rölla G. (1990) Six month study on the effect of a dentifrice containing zinc citrate and triclosan on plaque, gingival health and calculus. *Scand J Dent Res* 98:301-304.
6. Goodson J.M. (1989) Pharmacokinetic principles controlling efficacy of oral therapy. *J Dent Res* 68:1625-1632.
7. Cummins D. and Creeth J.E. (1992) Delivery of antiplaque agents from dentifrice, gels and mouthwashes. *Int Dent J* 71:1439-1449.
8. Lane R.M., Hull D.M.C., Saxton C.A. and van der Ouderaa F.J.G. (1985) Dentifrice Compositions. *Patent EP 0161 898.*
9. Grigor J. and Roberts A.J. (1992) Reduction in the levels of oral malodour precursors by hydrogen peroxide: in vitro and in vivo assessment. *J Clin Dent* 3:111-115.
10. Grigor J., Huntington E., Roberts A.J. and Tonzetich J. (1993) Evaluation of analysis of salivary thiol levels as a predictor of oral malodour. *J Dent Res* 72:272 (Abstr 1347).
11. Tonzetich J. (1971) Direct gas chromatographic analysis of sulphur compounds in mouth air in man. *Archs oral Biol* 16:587-597.
12. Rosenberg M., Septon I., Eli I., Bar-Ness R., Gelernter I., Brenner S. and Gabbay J. (1991) Halitosis measurement by an industrial sulphide monitor. *J Periodontol* 62:487-489.
13. De Boever E.H., De Uzeda M. and Loesche W.J. (1994) Relationship between volatile sulfur compounds, BANA-hydrolysing bacteria and gingival health in patients with and without complaints of oral malodour. *J Clin Dent* 4:114-119.

14. Niles H.P., Miller S. and Gaffar A. (1994) Breath freshening effects of a triclosan/copolymer/NAF dentifrice. *J Dent Res* 73:165 (Abstract).
15. Roberts A.J., Grigor J., Huntington E. and Tonzetich J. (1993) Reduction of Oral Malodour and its thiol precursors by mouthrinses containing soluble zinc complexes. *J Dent Res* 72:272 (Abstract).
16. Tonzetich J. (1978) Oral Malodour: an indicator of health status and oral cleanliness. *Int Dent J* 28:309-319.
17. Miyazaki H., Sakao S., Katoh Y. and Takehara T. (1995) Oral malodour in the general population of Japan. In: Rosenberg M. (Ed) *Bad Breath: Research Perspectives.* Ramot Publishing, Tel Aviv University, Tel-Aviv, Israel, pp 119-136.
18. Jones C.L., Ritchie J.A., Marsh P.D. and van der Ouderaa F.J.G. (1988) The effect of long term use of a dentifrice containing zinc citrate and a nonioninc agents in the oral cavity. *J Dent Res* 67:46-50.
19. Duckworth R. (1995) Personal communication.

The Effect of Tongue Brushing or Toothpaste Application on Oral Malodour Reduction

K. Hoshi and D. van Steenberghe[*]

Introduction

Volatile sulphur compounds (VSC) are accepted as important components of oral malodour (1, 2). Oral production of malodorous substances is associated with protein degradation performed by the bacteria covering the oral hard and soft tissues (3, 4, 5). Recent studies implicate the dorsum of the tongue as an important source of VSC (6). Bosy *et al.* (7) also reported that organoleptic judgement of tongue malodour is correlated with VSC level. Tonzetich and Ng (8) reported that combining tooth and tongue brushing was an effective technique to decrease oral malodour.

Triclosan is a broad-spectrum antibacterial agent which has been used for the past 20 years, mainly in deodorants, soaps, and body powders (9, 10, 11). The chemical name of triclosan is 2,4,4'-trichloro-2'-hydroxydiphenyl ether. The primary site of antimicrobial action of triclosan is the cytoplasmic membrane of bacteria. At bacteriostatic concentrations, triclosan prevents the uptake of essential amino acids. At bactericidal concentrations, triclosan causes disorganization of the cytoplasmic membrane of bacteria and leakage of cellular contents. Triclosan is effective against a variety of both Gram-positive and Gram-negative bacteria (12, 13, 11). A recent development is the introduction of triclosan for the reduction of plaque and gingivitis (14, 11, 15).

Mouthwashes containing zinc salts were first reported to reduce the growth of dental plaque in 1973 (16). The activity of zinc was confirmed in the work of Harrap *et al.* (17), which stressed the importance of using a high enough concentration of zinc and a sufficient frequency of application. Water-insoluble zinc salts were inactive, but the partially soluble zinc citrate gave activity *in vivo* similar to that of the readily soluble zinc phenolsulphonate. Toothpastes are more widely used as oral hygiene aids than mouthwashes, so it is desirable to deliver antiplaque activity from these aids. The inclusion of zinc citrate reduced the plaque growth in the study of dentifrices (18).

---

* K. Hoshi and D. van Steenberghe, Department of Periodontology, Faculty of Medicine, Catholic University Leuven, Capucijnenvoer 7, 3000 Leuven, Belgium.

The use of the combination of zinc and triclosan in toothpaste for the prevention of plaque and gingivitis is well documented (19, 20, 21). Therefore a toothpaste containing both zinc and triclosan was investigated to measure its potential to decrease oral malodour.

This study compared the effect of tongue brushing vs. tooth paste application on the tongue dorsum on VSC. Measurements were carried out using a portable sulphide monitor (Interscan Corp, Chatsworth, USA) according to a simple and rapid technique proposed by Rosenberg *et al.* (22, 23).

As shown in an animal study (24) the effect of triclosan is enhanced when it is delivered in a lipophilic gel formulation. *In vitro*, the plaque-inhibiting effect of triclosan is influenced by the combination with some flavouring oils (25). Therefore, a toothpaste which contains zinc citrate and triclosan without the flavour oil was specially made and its effectiveness to lower oral malodour was tested in this study.

Materials and Methods

Patient selection

Ten male volunteers aged 20-34 years were included in this study. Exclusion criteria were medication within the three months before or during the study, smoking, systemic diseases, the presence of dental caries and oral mucosal lesions.

Experimental design

Volunteers were examined four different mornings. A waiting period of more than two days elapsed between each morning. The subjects were instructed not to consume onion, garlic or alcohol at their last meal, and were instructed to brush their teeth without toothpaste. No tongue brushing was performed. Subjects were asked to refrain from eating, drinking, and any oral hygiene procedure during the four mornings. Perfumes or strong odour cosmetics were also not permitted.

VSC measurements were performed with a portable industrial sulphide monitor (Halimeter™, Model RH-17E, Interscan Corp., Chatsworth, USA). The monitor was adjusted to zero on ambient air before each measurement. A disposable flexible drinking straw was connected to the factory-supplied

tubing and inserted in the subjects' mouth, with the top of the straw 3 cm behind the incisors, while the mouth was kept slightly opened. Results were recorded as peak parts per billion sulphide equivalents. In total, 10 different time points of measurements were taken from early in the morning during four hours. On day 1, no treatment was performed during measurements. On day 2, tongue brushing with a normal tooth brush was performed just after the first measurement by scraping 10 times the tongue dorsum from the circumvallate papillae to the apex by the same professional. It was a standardized procedure for all subjects. On day 3, a commercial tooth paste (Signal™ Global, Unilever UK) with 0.75% zinc citrate, 0.3% triclosan, 0.7% to 1.6% flavour oil, and sodium lauryl sulphate as a anionic detergent, was applied. Tooth paste (1.0 g) was put on the tongue and mixed by tongue movements for 15 seconds. On day 4, an experimental tooth paste, which was identical (0.75% zinc citrate and 0.3% triclosan) to the previous one but without oil-based flavour material, was applied. After treatments on day 2, 3, and 4, oral rinsing was done twice with 50 ml tap water during 20 seconds.

Statistical analysis

At each time point, 5 consecutive VSC measurements were recorded. The mean value of these 5 measurements was used for further analysis. A two tailed t-test for paired data was used between the first measurement and the second (15 minutes later) and also between the first and the tenth measurement (4 hours later) to compare VSC values.

Results

VSC changes in the 10 subjects during the morning without treatment is shown in Fig. 1. For the first measurement, a large interindividual variation (from 57 ppb to 197 ppb) is observed. When comparing VSC values, no significant difference appears between the baseline and after 15 minutes. There is also no significant difference between the baseline and the measurement at 4 hours. VSC changes after tongue brushing are shown in Fig. 2. There is a significant difference between baseline and the 15 minutes values. There is no significant difference between the baseline and the 4 hours measurement. VSC changes after the commercial tooth paste application are shown in Fig. 3. There are significant differences between the baseline and 15 minutes and also at 4 hours. In Fig. 4, VSC changes with

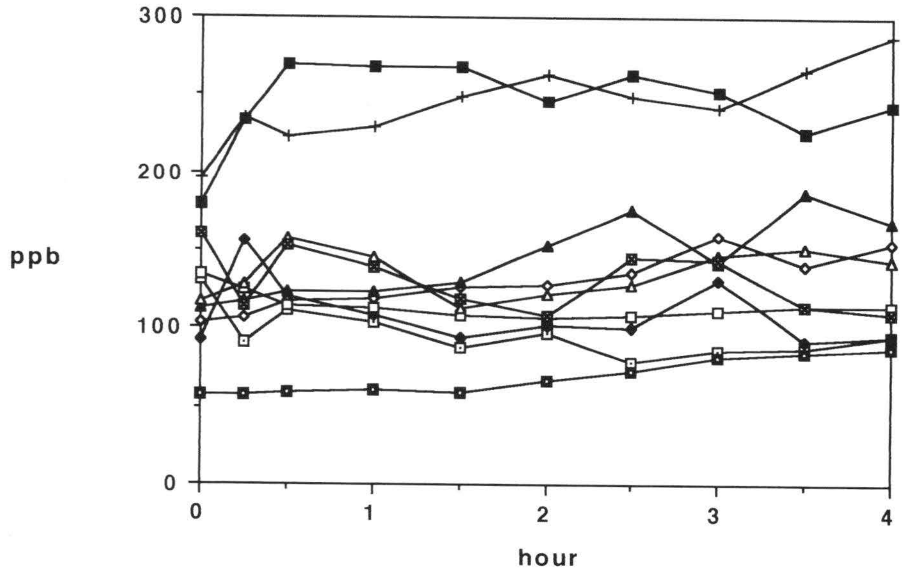


Fig. 1 : Morning VSC levels in 10 fasting subjects without treatment.

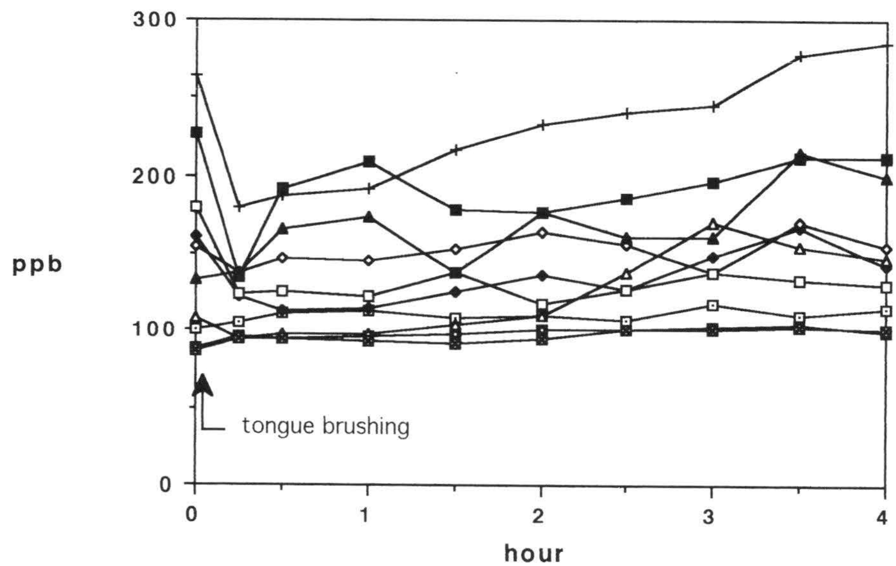


Fig. 2 : Morning VSC levels in 10 subjects after tongue brushing without toothpaste.

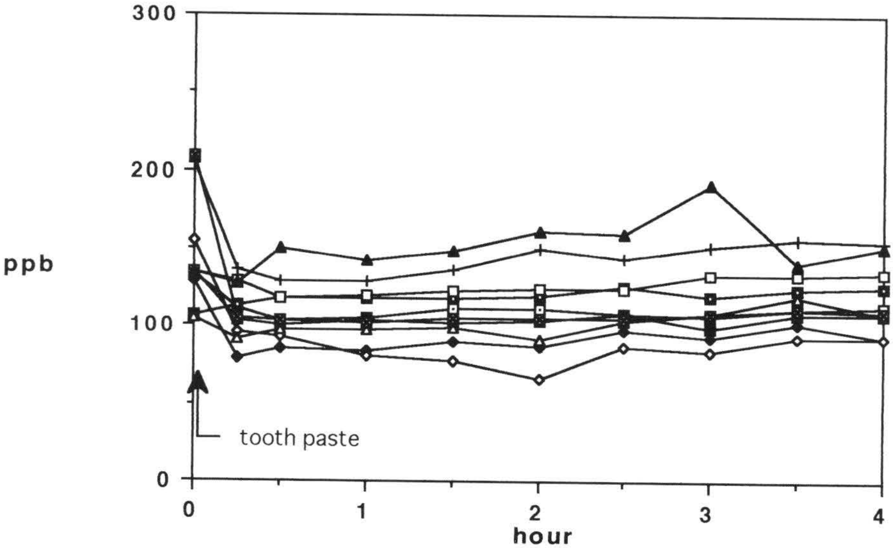


Fig. 3 : Morning VSC levels in 10 subjects after application of a commercial toothpaste containing zinc and triclosan on the tongue dorsum.

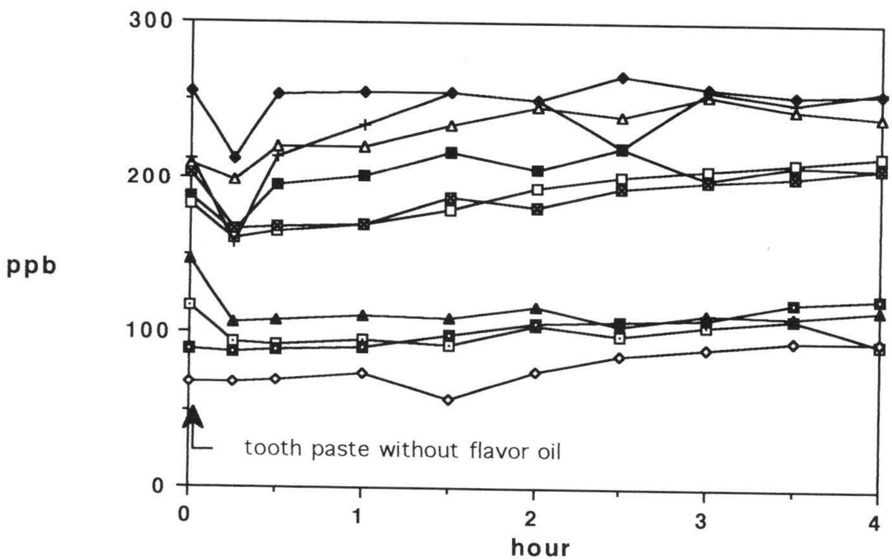


Fig. 4 : Morning VSC levels in 10 subjects after application of a toothpaste containing zinc and triclosan without the flavour oil on the tongue dorsum.

the specially made tooth paste containing zinc citrate and triclosan but no flavour oil is shown. Although a significant decrease was observed after 15 minutes, this was lost 4 hours later. The p-values compared with the first measurement are shown in Table 1.

Table 1 : Statistical analysis of VSC at 15 minutes and 4 hours compared with baseline

	p value	
treatment	15 min	4 hr
none	0.50	0.18
tongue brushing with water	0.04	0.44
toothpaste application	0.01	0.06
toothpaste application (without flavour oil)	0.002	0.12

Discussion

Some researchers have reported the gradual increase of VSC after meals (26) or prolonged periods of food intake and oral hygiene measures (2). VSC do not seem to increase during four hours in the fasting situation in this study probably because of the lack of food intake in the morning.

There is ample evidence that tongue coating plays a major role in oral malodour (8, 7). Thus tongue brushing seems a logical approach to decrease oral malodour, even if it is more or less impossible to reach the distal part of the tongue dorsum. The result of tongue brushing appears very short-lived since after 1.5 hour the VSC levels are back to baseline. The VSC value after 1.5 hour was not significant different from baseline value (p=0.419).

The mechanism of action of zinc is not established unequivocally. Binding of zinc ions to the surface of oral bacteria can alter their surface potential (27, 28) and this may affect bacterial adhesion to oral tissues (29). Additionally, because of the ability of zinc ions to combine with sulphur compounds (30), it may decrease odoriferous sulphur gases.

The mode of the antibacterial action of triclosan is not entirely clear. Presumably the hydrophobic portion of the triclosan molecule adsorbs to the lipid portion of the bacterial cell membrane and thus interferes with vital cell functions such as transmenbrane transportation. Oral bacteria such as *mutans Streptococci, Streptococcus sanguis* and *S. salivarius* are susceptible to low concentrations of triclosan *in vitro* (31), however those

bacteria were not on the list of heavy producers of VSC (32, 33, 34, 35, 36, 5).

Salivary triclosan concentration was 20 µl/ml 5 min after brushing with a dentifrice (0.3% triclosan dentifrice, 1g, 1 min brush). Triclosan remains present in plaque for at least 8 hours and on the oral mucosa for at least 3 hours after brushing (37, 38). For triclosan in a zinc citrate mixture, the biologic half-life was calculated to be 20 min (0.2% triclosan dentifrice, 1g, 1 min brush) (39). A 0.2% triclosan plus 0.5% zinc citrate toothpaste also reduces plaque regrowth over 16 hours (18). In the present study this toothpaste reduced VSC levels for at least 4 hours. This result thus justified the investigation into the possible therapeutic benefits of using this dentifrice.

Further enhancement of toothpaste activity, already provided by some of the detergents used, would appear so far quite a difficult task, as suggested by Jenkins *et al.* (21).

One problem of testing the activity of triclosan is its lipophilic nature (30, 25). Triclosan tends to partition into a hydrophobic oil phase and a surfactant micellar phase and is thereby rendered unavailable for adsorption onto the oral surfaces. Since the oil/micellar phases in a toothpaste are conventionally provided by flavouring oils (such as peppermint or spearmint) and anionic detergents (such as sodium lauryl sulphate) their removal as was done here for experimental purposes may be accompanied by a decreased bioavailability.

This can explain the surprising finding that the toothpaste specially prepared for this study, although containing zinc and triclosan, only had a shortlived effect.

In commercial products, triclosan is solubilized in one or more detergents, such as sodium dodecyl sulfate (SDS) and sodium lauroyl sarcosinate, or in propylene glycol or polyethylene glycol. *In vivo*, the antiplaque efficacy and the substantivity of triclosan alone are limited (40, 41). Therefore, great efforts have been made by the manufactures to enhance the *in vivo* efficacy of triclosan. Two different approaches have been pursued. One is to increase the efficacy by increasing the oral retention and decreasing the rate of release of triclosan by adding a copolymer, polyvinylmethylether maleic acid, to the formulation (PVM/MA) (42). The other is to potentiate the antimicrobial effect of triclosan by adding zinc citrate (18).

We conclude that the effect of tongue brushing on VSC is only short-lived. A commercially available toothpaste containing zinc citrate and triclosan remains effective in this perspective even after 4 hours. Removal of the flavour oil from this same tooth paste decreases the bioavailability of the active ingredients and compromises their efficiency in lowering VSC concentration.

Acknowledgements

We are grateful to Unilever Research, Port Sunlight Laboratory, UK, for providing the specially prepared toothpaste without flavour oils.

References

1. Tonzetich J. (1971) Direct gas chromatographic analysis of sulphur compounds in mouth air in man. *Archs oral Biol* 16:587-597.
2. Tonzetich J. (1977) Production and origin of oral malodor: A review of mechanisms and methods of analysis. *J Periodontol* 48:13-20.
3. McNamara T.F., Alexander J.F., Lee M. and Plains M. (1972) The role of microorganisms in the production of oral malodor. *Oral Surg Oral Med Oral Pathol* 34:41-48.
4. Kleinberg I. and Westbay G. (1990) Oral malodor. *Crit Rev Oral Biol Med* 1:247-259.
5. Persson S., Edlund M-B., Claesson R. and Carlsson J. (1990) The formation of hydrogen sulfide and methyl mercaptan by oral bacteria. *Oral Microbiol Immunol* 5:195-201.
6. Yaegaki K. and Sanada K. (1992) Biochemical and clinical factors influencing oral malodor in periodontal patients. *J. Periodontol* 63:783-789.
7. Bosy A., Kulkarni G.V., Rosenberg M. and McCulloch C.A.G. (1994) Relationship of oral malodor to periodontitis: evidence of independence in discrete subpopulations. *J Periodontol* 65:3 746.
8. Tonzetich J. and Ng S.K. (1976) Reduction of malodor by oral cleansing procedures. *Oral Surg Oral Med Oral Pathol* 42:172-181.
9. Ciancio S.G. (1992) Agents for the management of plaque and gingivitis. *J Dent Res* 71:1450-1454.
10. Webster J. (1992) Handwashing in a neonatal intensive care nursery: product acceptability and effectiveness of chlorhexidine gluconate 4% and triclosan 1%. *J Hosp Infect* 21:137-141.
11. Volpe A.R., Petrone M.E., DeVizio W. and Davies R.M. (1993) A review of plaque, gingivitis, calculus and caries clinical efficacy studies with a dentifrice containing triclosan and PVM/MA Copolymer. *J Clin Dent* 4:31-41.
12. Furia J.E. and Schenkel A.G. (1968) New, broad spectrum bacteriostat. *Soap and Chemical Specialities* 47-50:116-121.
13. Regos J. and Hitz H.R. (1974) Investigations on the mode of action of triclosan, a broad spectrum antibacterial agent. *Zbl Bakt Hyg*

226:390-401.
14. Wade W.G. and Addy M. (1992). Antibacterial activity of some triclosan containing toothpastes and their ingredients. *J Periodontol* 63:280-282.
15. Renvert S. and Birkhed D. (1995) Comparison between 3 triclosan dentifrices on plaque, gingivitis and salivary microflora. *J Clin Periodontol* 22:63-70.
16. Fischman S.L., Picozzi A., Cancro L.P. and Pader M. (1973) The inhibition of plaque in humans by two experimental oral rinses. *J Periodontol* 44:100-102.
17. Harrap G.J., Saxton C.A. and Best J.S. (1983) Inhibition of plaque growth by zinc salts. *J Periodont Res* 18:634-642.
18. Saxton C.A., Harrap G.J. and Lloyd A.M. (1986) The effect of dentifrices containing zinc citrate on plaque growth and oral zinc levels. *J Clin Periodontol* 13:301-306.
19. Saxton C.A. (1986) The effect of a dentifrice containing zinc citrate and 2,4,4' trichloro-2"-hydroxydiphenyl ether. *J Periodontol* 57:555-561.
20. Saxton C.A., Lane R.M. and van der Ouderaa F. (1987) The effect of a dentifrice containing a zinc salt and a non-cationic antimicrobial agent on plaque and gingivitis. *J Clin Periodontol* 14:144-148.
21. Jenkins S., Addy M. and Newcombe R. (1989) Studies on the effect of toothpaste rinces on plaque regrowth (II). Triclosan with and without zinc citrate formulations. *J Clin Periodontol* 16:385-387.
22. Rosenberg M., Septon I., Eli I., Bar-Ness R., Gelernter I., Brenner S. and Gabbay J. (1991) Halitosis measurement by an industrial sulphide monitor. *J Periodontol* 62:487-489.
23. Rosenberg M., Kulkarni G.V., Bosy A. and McCulloch C.A.G. (1991) Reproducibility and sensitivity of oral malodor measurements with a portable sulphide monitor. *J Dent Res* 70:1436-1440.
24. Mühlemann H.R. (1973) Chlorhexidine, vantocil, fluorophene and antimycotic agents in an animal caries test. *Helv Odontol Acta* 17:99-103.
25. Kjaerheim V., Waaler S.M. and Rölla G. (1994) Organic solvents and oils as vehicles for triclosan in mouthrinses: a clinical study. *Scand J Dent Res* 102:306-308.
26. Rosenberg M. and McCulloch C.A.G. (1992) Measurement of oral malodor: current methods and future prospects. *J Periodontol* 63:776-782.
27. Olsson J. and Glantz P.O. (1977) Effect of pH and counter ion on the zetapotential of oral streptococci. *Archs oral Biol* 22:461-466.
28. Olsson J. and Odham G. (1978). Effect of inorganic ions and surface active organic compounds on the adherence of oral streptococci. *Scand*

J Dent Res 86:108-117.

29. Skjörland K., Gjermo P. and Rölla G. (1978) Effect of some polyvalent cations on plaque formation in vivo. *Scand J Dent Res* 8 6:103- / 2-107.
30. Scheie A. (1994) Chemoprophylaxis of dental caries. In: Thylstrup A. and Fejerskov O. (Eds.) *Textbook of Clinical Cariology*. Munksgaard, Copenhagen, Denmark, pp 311-326.
31. DeSalva S. J., Kong B.M. and Lin Y.J. (1989) Triclosan: a safety profile *Am J Dent* 2:185-196.
32. Dzink J.L., Tanner A.C.R., Haffajee A.P. and Socransky S.S. (1985) Gram negative species associated with active destructive periodontal lesions. *J Clin Periodontol* 12:648-659.
33. Dzink J.L., Socransky S.S. and Haffajee A.D. (1988) The predominant cultivable microbiota of active and inactive lesions of destructive periodontal diseases. *J Clin Periodontol* 15:316-323.
34. Loesche W.J. and Laughon B.E. (1982) Role of spirochetes in periodontal disease. In: Genco R.I. and Mergenhagen S.E. (Eds.) *Host-parasite interactions in periodontal diseases.* Washington, DC: Am Soc Microbiol, pp 62-75.
35. Slots J (1986) Bacterial specificity in adult periodontitis-a summary of recent work. *J Clin Periodontol* 13:912-917.
36. Slots J. and Listgarten M.A. (1988) *Bacteroides gingivalis, Bacteroides intermedius* and *Actinobacillus actinomycetemcomitans* in human periodontal diseases. *J Clin Periodontol* 15:85 -93.
37. Afflitto J., Fakhry-Smith S. and Gaffar A. (1989) Salivary and plaque triclosan levels after brushing with a 0.3 per cent triclosan/ copolymer/Naf dentifrice. *Am J. Dent* 2:207-210.
38. Cummins D. (1991) Zinc citrate/triclosan:a new anti-plaque system for the control of plaque and the prevention of gingivitis: short term clinical and mode of action studies. *J Clin Periodontol* 18:453-461.
39. Gilbert R.J. (1987) The oral clearance of zinc and triclosan after delivery from a dentifrice. *J Pharm Pharmacol* 39:480-483.
40. Jenkins S., Addy M. and Newcombe R. (1991) Triclosan and sodium lauryl sulphate mouthwashes. (I). Effects on salivary bacterial counts. *J Clin Periodontol* 18:140-144.
41. Jenkins S., Addy M. and Newcombe R. (1991). Triclosan and sodium lauryl sulphate mouthrinses. (II). Effects of 4-day plaque regrowth. *J Clin Periodontol* 18:145-148.
42. Nabi N., Mukerjee C., Schmid R. and Gaffar A. (1989) In vitro and in vivo studies on triclosan/PVM/MA copolymer/NaF combination as an anti-plaque agent. *Am J Dent* 2:197-206.

Development and Testing of a Combined Physical and Chemical Approach to Treating Halitosis

*O. Ilan, A. Kozlovsky, S. Goldberg, E.I. Weiss, and M. Rosenberg**

Most cases of bad breath originate in the mouth itself, as the result of microbial putrefaction of proteinaceous oral debris and secretions (1). Based on this observation, several treatment strategies may be considered. One is to reduce the numbers of viable odorigenic microorganisms in the mouth, or to inhibit their metabolic activity (2). Secondly, the level of available nutrients, especially the particulate matter considered to be critical for malodour production (3), can be limited. Thirdly, strategies can be considered for interfering with production and volatilization of the malodorous products themselves (see Raven *et al.,* this text).

In general, these strategies are undertaken using either mechanical or chemical approaches, or a combination of the two. Examples of mechanical treatments include toothbrushing, tongue cleaning, water jets and interdental cleaning. Various methods are available for cleaning the tongue, e.g. brushing with a regular or specially-designed brush, scraping using tonguescrapers or spoons, and wiping with gauze. Dental floss and anatomic tooth picks are often used for interdental cleaning.

Most chemical approaches to combatting oral malodour involve mouthwashes. Mouthrinsing is a common oral hygiene practice dating back to ancient times (4). Components considered to be active include antibacterial compounds, include cetylpyridinium chloride, chlorhexidine, triclosan, essential oils, zinc salts and combinations thereof. Oxidizing agents (e.g., chlorine dioxide) are incorporated in several current anti-malodour rinses, although their efficacy has yet to be established scientifically.

Much of the evidence for the efficacy of mouthrinses in reducing bad breath is anecdotal (5), and there are very few publications in the scientific literature on this subject. Long term malodour reduction (over three hours) has rarely been addressed. For example, in investigations carried out by Pitts *et al.* (6), Schmidt and Tarbet (7), and Tonzetich (8), data on malodour reduction were collected up to three hours following use.

---

* O. Ilan[1,2], A. Kozlovsky[1], S. Goldberg[1,2], E. Weiss[1] and M. Rosenberg[1,2], The Maurice and Gabriela Goldschleger School of Dental Medicine[1], and the Department of Human Microbiology[2], Sackler Faculty of Medicine, Tel-Aviv University, Ramat-Aviv 69978, Israel.

Two Phase Mouthwash Development

Over the past thirteen years, much of our interest in treatment of oral malodour and not debris has focused on the potential of two-phase, oil/water mixtures to remove oral bacteria and to combat bad breath. Our initial finding was that a high proportion of oral bacteria adhere to oil droplets. In 1982, Weiss *et al.* (9) reported that most oral microbial isolates retrieved from extracted teeth adhered to the liquid hydrocarbon, hexadecane. It was subsequently demonstrated that washed, dispersed suspensions of microorganisms obtained directly from supragingival dental plaque adhered in high proportions to droplets of liquid hydrocarbon (10). These initial observations led to the notion of using two-phase oil/water mouthwashes for physical desorption of oral microbiota. Indeed, other studies have demonstrated that a wide variety of laboratory strains of oral microorganisms, including *Actinobacillus actinomycetemcomitans, Actinomyces viscosus* and *Actinomyces naeslundii, Porphyromonas gingivalis, Candida albicans, Streptococcus salivarius*, and certain *Streptococcus mutans* strains, adhere with high affinity to hydrocarbons (for a review, see Doyle *et al.* (11)). Initial experiments showed that microorganisms which adhere to hydrocarbons also bind to other nontoxic oils (12). Furthermore, *in vitro* experiments showed that water/oil combinations could desorb bacteria from plastic surfaces (12,13). Desorption could also be demonstrated *in vivo*, in the following experiment: a subject stained the interior of his mouth by rinsing with erythrosine. Following sequential saline rinses in order to wash out residual stain, the subject rinsed three times with a mixture of saline and olive oil. Following phase separation of the expectorates, it was evident that desorbed bacteria and other stained oral debris, which had not been removed with the previous saline rinses, had been removed onto the surface of oil droplets (Fig. 1). Following the aqueous/oil rinses, a final saline rinse was performed, with negligible removal of stain. This and other similar experiments demonstrated that the two phase oil/water combination was clearly superior in oral bacteria and debris desorption, as compared with water alone.

Despite these initial desorption experiments, the initial aqueous/oil combinations did not prove particularly effective in combatting oral malodour. Furthermore, combining the two immiscible oil and water phases posed technical difficulties. In 1991, Goldberg and coworkers (14) found that adhesion of microorganisms and oral debris to oil was promoted by low concentrations of cetylpyridinium chloride (CPC), an amphipathic cationic agent which is commonly added to mouthwashes. This was surprising, since amphipathic agents were previously considered to inhibit adhesion at the

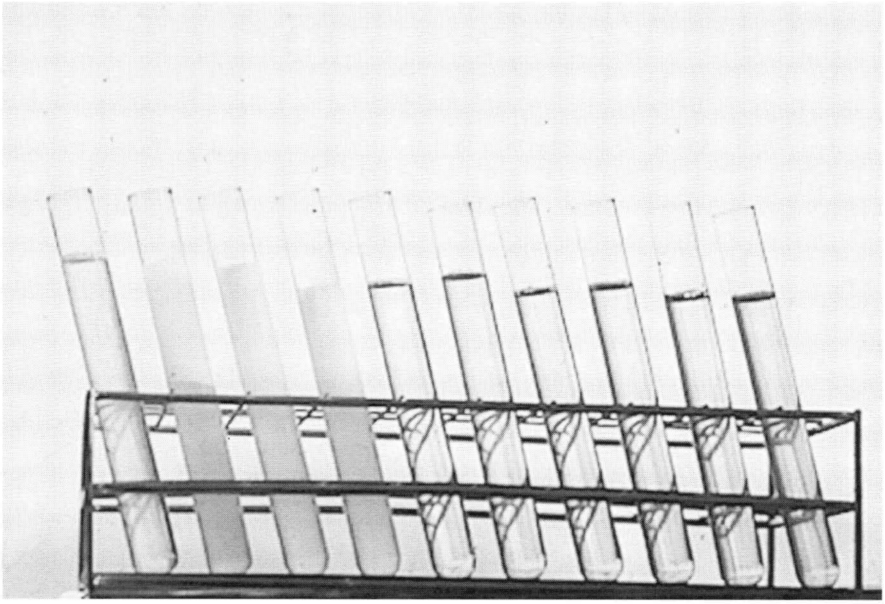


Fig. 1 : Desorption of bacteria as achieved by the two-phase mouthwash.

oil/water interface (15). Since CPC is an antibacterial agent, its addition had several additional advantages, including inhibition of microbial activity in the mouth, and increasing shelf life of the product by preventing microbial outgrowth. Furthermore, addition of an amphipathic agent such as CPC to oil/water mixtures results in a temporary emulsion following shaking. This in turn enabled the mixture to be supplied in a single bottle.

Prototype formulations, consisting of an aqueous phase containing CPC, and an oil phase comprising a mixture of vegetable and essential oils, were subsequently developed and tested. Such formulations maintained dramatic *in vitro* bacterial desorption properties, removing over 90% of a bacterial film, as compared to 15-50% removal for a series of commercial mouthwashes (14).

In an initial clinical study, the prototype two-phase rinse was compared with (i) a placebo rinse; and (ii) a mouthrinse containing 0.2% chlorhexidine gluconate (Corsodyl™ , ICI, England). Subjects performed oral rinsing prior to bedtime and in the morning of the following day. Measurements were taken in the late afternoon (e.g. at least eight hours following rinsing) of the second day and were compared to those taken the previous day afternoon prior to

rinsing. The data demonstrated that rinsing with the two-phase mouthwash results in daylong reduction in bad breath-associated parameters (16). In another study employing the same two phase formulation, Yaegaki and Sanada (3) showed dramatic reductions in volatile sulphide levels 3.5 hours after use, as compared to a commercial mouthwash (Skoal™). These authors suggested that physical removal of the particulate oral debris by the mouthrinse was an important factor in malodour reduction.

In 1992, the prototype two-phase oil-water mouthwash was reformulated and introduced in the Israeli market. A clinical study (17) was performed to compare the efficacy of the commercial version of the two-phase mouthwash with Listerine™, a mouthrinse which has been previously shown (6,18) to be effective in reducing levels of odor-related microorganisms, as well as plaque and gingivitis. In the study, fifty subjects rinsed with one of the two mouthwashes for 30 seconds twice a day over six weeks, while continuing their regular oral hygiene habits. In both mouthwash groups, dramatic improvements were observed in parameters associated with malodour, periodontal health, plaque accumulation, and microbial levels. As compared to time zero scores, whole mouth odour, tongue dorsum anterior and posterior odours decreased continuously over time, attaining 80%, 79% and 70%, reductions, respectively following 6 weeks, in the two-phase mouthwash group, vs. 70%, 77% and 59% for the Listerine™ group. For whole mouth and tongue dorsum posterior, the reductions observed in the two-phase mouthwash group were significantly greater than those obtained with Listerine™ ($p=0.026$ and $p=0.025$, respectively). These data suggest that the two-phase mouthwash is superior to Listerine™ in long-term reduction of oral malodour.

Slow Release of Antibacterial Acitivy from Oil Droplets

The potent anti-malodour and antibacterial efficacy of the two-phase CPC-containing mouthrinse suggested that mechanisms other than desorption of bacteria and debris might also be involved. One possibility is that the presence of the oil droplets somehow enhance the antibacterial activity of the CPC, a compound which generally shows limited substantivity (19).

In order to test whether the antimicrobial effect of the CPC is enhanced in the presence of oil droplets, we performed several types of experiment. In the first, a temporary emulsion was obtained by brief mixing of a suspension consisting of (i) 0.05% CPC in double distilled water (80% v/v), and (ii) oil

(hexadecane; 20% v/v). Each individual sample (2.2 mL) was mixed by pumping down and up with a disposable polypropylene pipettor (2 mL, Sterilin, England) in a test tube. The emulsion was then sucked back into a clean tip attached to a pipette (pipet-aid, Drummond Scientific Co., Japan) and immediately applied to the bottom of a clean glass test tube (Kimble, 12x75mm), and removed by suction using the same tip. To the residue, growth medium (2 mL, brain heart infusion broth, Difco Ltd., Ann Arbor, Mich) containing a dilution of a stationary phase culture of *Escherichia coli* to yield 0.02 O.D. (400 nm, 1 cm cell path), was added. Test tubes were incubated for varying times at 37°C with no shaking, to test for residual antimicrobial activity. Microbial outgrowth was ascertained by optical density readings at 400 nm over time. As control, test tubes were similarly treated with double distilled water (DDW).

Results are summarized in Fig. 2. Hexadecane alone had essentially no inhibitory effect as compared to the control with no additions. CPC alone had an inhibitory effect for the first 3 hours, which was followed by rapid outgrowth. In contrast, the test tubes containing the residue of the temporary emulsion (CPC + hexadecane) provided dramatic inhibition even after 18 hours. One possible explanation for this is that more CPC-containing residue was left in

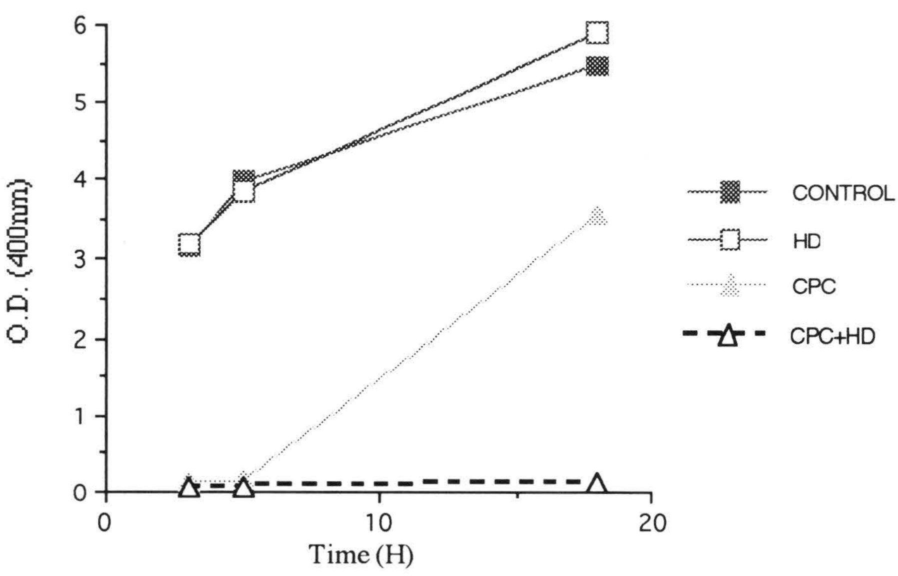


Fig. 2 : Outgrowth of E. coli in test tubes treated with CPC+hexadecane, as opposed to CPC and hexadecane alone. Results are expressed as optical density as a function of time of incubation.

the test tubes in the case of the temporary emulsion. Evidence in favour of this premise could be obtained by simply weighing the test tubes (Table 1). A significantly greater amount of residue remained in the test tubes when both CPC and hexadecane were present, as compared with either CPC alone ($p < .005$) or hexadecane alone ($p < 0.05$).

Table 1 : Weight of Residue Left in Test Tubes

Addition	Weight of Residue[a]
Control	11.7 ± 0.7 mg
HD	18.8 ± 6.5 mg
CPC	14.6 ± 2.6 mg
CPC + HD	31.5 ± 4.9 mg

[a] *Mean of 4 test tubes ± standard deviation*

In order to more closely approximate the oral cavity, we also measured the residual antimicrobial activity following application of the oil/water mixture to extracted teeth. Crowns of eight extracted human wisdom teeth were sectioned using a conventional dental handpiece and diamond disc. Each crown was then sectioned into four equivalent quarters. Crown sections were divided into groups of four according to their size, so that all crowns within a given group would have similar surface areas. All sections were immersed in phosphate-buffered saline (8.8g NaCl, 0.26g

NaH_2PO_4, 1.2g Na_2HPO_4 and distilled water to 1 liter [pH 7.4]) and sterilized by autoclaving. Sections were washed thoroughly in DDW and dried. Sections were immersed for one second into an emulsion consisting of (i) 0.05% CPC in DDW (80% v/v), and (ii) hexadecane (20% v/v). Control sections were immersed for one second into either 0.05% CPC in double distilled water or into hexadecane. Sections were then applied briefly to absorbent paper to remove excess liquid, and then placed into glass test tubes containing sufficient liquid (brain heart infusion broth medium seeded with *E. coli* at an initial O.D. of 0.02) to cover the sections (0.5 ml). Test tubes were incubated at 37°C, to test for residual antimicrobial activity. Microbial outgrowth was ascertained by optical density readings at 400 nm (1 cm cell path) following overnight incubation.

The results are presented as the relative outgrowth, compared to the control crown sections which were immersed in DDW (Table 2).

Table 2 : Relative inhibition of outgrowth (overnight) following immersion of teeth sections in CPC, hexadecane, or CPC + hexadecane

Tooth sections immersed in	Percent Inhibition of Outgrowth[a]
double distilled water	-
hexadecane	4%
CPC	9%
CPC + hexadecane	24%

[a] *As compared to water control; mean of eight samples.*

Tooth sections to which the temporary emulsion containing CPC and hexadecane were applied had a greater inhibitory effect (24%) than either or both CPC or hexadecane alone (9% and 4%, respectively).

In addition to trapping of interstitial water containing CPC around and between droplets on surfaces, it is also possible that amphipathic cations can be released over time from the surface of the oil droplets themselves. To study the release of amphipathic cationic antibacterial agents from the droplets, the following experiment was performed. Temporary emulsions were obtained by mixing a suspension consisting of (i) 0.05% antibacterial agent (CPC or benzalkonium chloride [BKC]) in DDW (4 mL), and (ii) 1 mL of oil phase. Each individual sample (5 mL) was mixed by pumping down and up with a disposable polypropylene pipettor (1 mL) in a test tube for 15 sec. The experiment was performed in duplicate. At different times following mixing, the concentration of the antibacterial agent in the aqueous lower phase was determined spectrophotometrically by optical absorbance at 254 nm for CPC and 263 nm for BKC.

Results are presented in Fig. 3. Results are expressed as percent free antibacterial agent, compared with the initial pre-mixing concentration (0.04%).

The results show that the oil droplets release the antibacterial agent back into the aqueous phase over time, showing that the oil droplets may act as a mechanism for releasing cationic antibacterial agents over time.

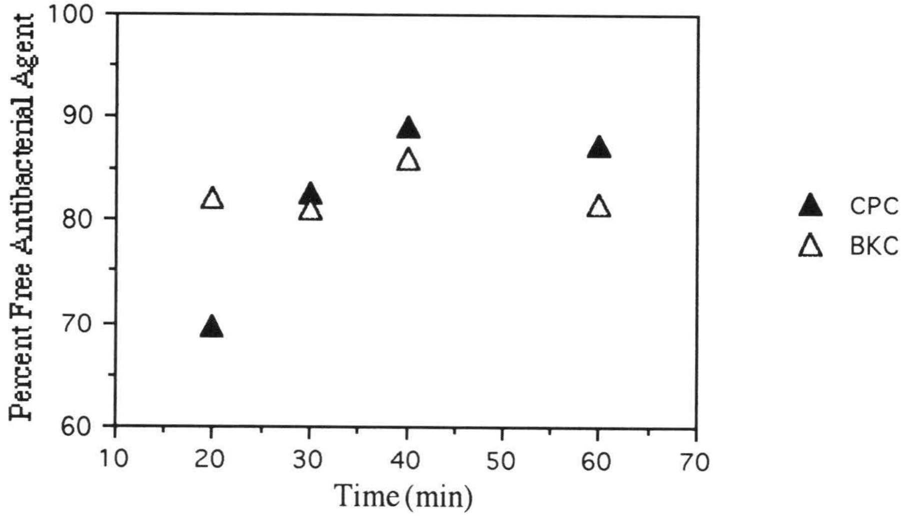


Fig. 3 : Release of CPC and BKC from oil droplets over time.

Conclusions

In general, current mouthrinses depend on their activity on a combination of physical desorption and cleaning, combined with chemical treatment. Rinsing in the absence of antibacterial agents (e.g., with water) results in only a transient (approximately 15 minute) reduction in oral malodour (1), whereas efficacious mouthrinsing with chemical agents (e.g., 0.2% chlorhexidine) results in malodour reductions for eight hours and more (16,17). We have previously shown that rinsing with oil/water formulations containing CPC have greater desorptive ability than aqueous formulations (14). More recently, the long term anti-malodour effect of a commercial version has been demonstrated (17). From the *in vitro* experiments described here, it appears that the oil droplets may remain in the oral cavity and effect a slow release of CPC, either from the surface of the droplets, or from the water immediately surrounding them. This mechanism of enhancing the *in vivo* substantivity of CPC may help explain the clinical efficacy of the two-phase mouthrinse in long term reduction of microbial levels.

Since the clinical experiments described above (17) were completed, triclosan has been added to the oil phase of the current Israeli commercial product (Assuta™). Although no studies have been conducted to test whether even greater efficacy is thus obtained, it is possible that oil droplets persisting in the mouth may release triclosan over time, in addition to CPC.

References

1. Tonzetich J. (1977) Production and origin of oral malodor: A review of mechanisms and methods of analysis. *J Periodontol* 48:13-20.
2. Perdok J.F., Van der Mei H.C. and Busscher H.J. (1989) A comparison of bacterial growth inhibition effects of six commercially available mouthrinses. *Microb Ecol Health Dis* 2:191-196.
3. Yaegaki K., and Sanada K. (1992) Effects of a two-phase oil-water mouthwash on halitosis. *Clin Prevent Dent* 14:5-9.
4. Mandel I.D. (1988) Chemotherapeutic agents for controlling plaque and gingivitis. *J Clin Periodontol* 15:488-498.
5. Dever J.G. (1979) Oral hygiene in mentally handicapped children. A clinical trial using a chlorhexidine spray. *Australian Dent J* 24:301-305.
6. Pitts G., Brogdon C., Hu L., Masurat T., Pianotti R. and Schumann P. (1983) Mechanism of action of an antiseptic, anti-odor mouthwash. *J Dent Res* 62:738-742.
7. Schmidt N.F. and Tarbet W.J. (1978) The effect of oral rinses on organoleptic mouth odor ratings and levels of volatile sulfur compounds. *Oral Surg* 45:876-883.
8. Tonzetich J. (1977) Oral malodor: an indicator of health status and oral cleanliness. *Int Dent J* 28:309-319.
9. Weiss E., Rosenberg M., Judes H. and Rosenberg E. (1982) Cell-surface hydrophobicity of adherent oral bacteria. *Curr Microbiol* 7:125-128.
10. Rosenberg M., Judes H. and Weiss E. (1983) Cell surface hydrophobicity of dental plaque microorganisms in situ. *Infect Immun* 42:831-834.
11. Doyle R.J., Rosenberg M. and Drake D. (1990) Hydrophobicity of oral bacteria. In: Doyle R.J. and Rosenberg M. (Eds) *Microbial Cell Surface Hydrophobicity*, ASM Washington, D.C. pp 387-419.
12. Rosenberg M., Judes H. and Weiss E. (1983) Desorption of adherent bacteria from a solid hydrophobic surface by oil. *J Microbiol Methods* 1:239-244.
13. Bar-Ness R. and Rosenberg M. (1989) Putative role of a 70 kda surface protein in mediating cell surface hydrophobicity of *Serratia marescens*. *J Gen Microbiol* 135:2277-2281.
14. Goldberg S. and Rosenberg M. (1991) Bacterial desorption by commercial mouthwashes vs. two-phase oil/water formulations. *Biofouling* 3:193-198.
15. Rosenberg M. (1984) Bacterial adherence to hydrocarbons: a useful technique for studying cell surface hydrophobicity. *FEMS Microbiol*

Letters 22:289-295.

16. Rosenberg M., Gelernter I., Barki M. and Bar-Ness R. (1992) Daylong reduction of oral malodor by a two-phase oil/water mouthrinse, as compared to chlorhexidine and placebo rinses. *J Periodontol* 63:39-43.
17. Kozlovsky A., Goldberg S., Natour I., Rogatky-Gat A., Gelernter I. and Rosenberg M. (1996) Efficacy of Assuta® mouthwash in controlling mouth odor, gingivitis and plaque, in comparison with Listerine®. *J Periodontol* 67:577-582.
18. DePaola L.G., Overholser C.D., Meiller T.F., Minah G.E. and Niehaus C. (1989) Chemotherapeutic inhibition of supragingival dental plaque and gingivitis development. *J Clin Periodontol* 16:311-315.
19. Vandekerckhove B.N.A., van Steenberghe D., Tricio J. and Rosenberg D. (1995) Efficacy on supragingival plaque control of cetylpyridinium chloride in a slow-release dosage form. *J Clin Periodontol* 22:824-829.

Deodorizing Mechanism of Epigallocatechin and Chewing Gum Containing Tea Extracts

*M. Tsunoda, S. Yamada and H. Yasuda**

The effect of green tea on halitosis has been well known from early times. We have previously reported the inhibitory activity of tea catechins against methyl mercaptan (CH_3SH). Among the tea catechins epigallocatechin (EGCg) had the most deodorizing activity against CH_3SH.

The aim of this study was to investigate the deodorizing mechanism of EGCg and the effect of chewing gum containing tea extracts for halitosis.

The deodorizing activity of each sample against CH_3SH was assayed by gas chromatographic analysis of the headspace gas. The deodorizing effect of tea catechins as EGCg, (-)-epicathechin gallate (ECg), (-)-epicatechin (EC), (-)-epigallocathecin (EGC) and of gallic acid (GA) against CH_3SH was evaluated (Fig. 1).

The natural deodorants such as tea catechin, green tea extract, black tea extract, copper chlorophyl and sodium copper chlorophyllin had high deodorant activity by the experimental use of saliva of patients with periodontitis (Table 1). CH_3SH in the mouth air at 5 minutes after mouth-washing with the garlic extract solution was about 3ng/5ml, whereas by chewing of gum for 5 minutes CH_3SH was reduced to below 1.1 ng/5ml. The effect depended on the concentration of the deodorants in chewing gum.

Table 1 : Inhibitory action of deodorizing compounds against CH_3SH

Compounds	Control	Add 1 mg		Add 5 mg		Add 10 mg	
	VSC	VSC	Inhibitory rate	VSC	Inhibitory rate	VSC	Inhibitory rate
Tea catechin	41.80	1.78	95.7%	0.13	99.7%	0	100%
Green tea extracts	23.80	0.54	97.8%	0.05	99.8%	0	100%
Black tea extracts	28.40	0.13	99.5%	0	100%	0	100%
Copper chlorophyl	36.00	0.13	99.6%	0	100%	0	100%
Sodium copper chlorophyllin	37.80	3.12	92.5%	0.12	99.7%	0	100%

* M. Tsunoda, S. Yamada and H. Yasuda*, Department of Periodontics, Tokyo Dental College, * Lotte Central Laboratory Co, Ltd.

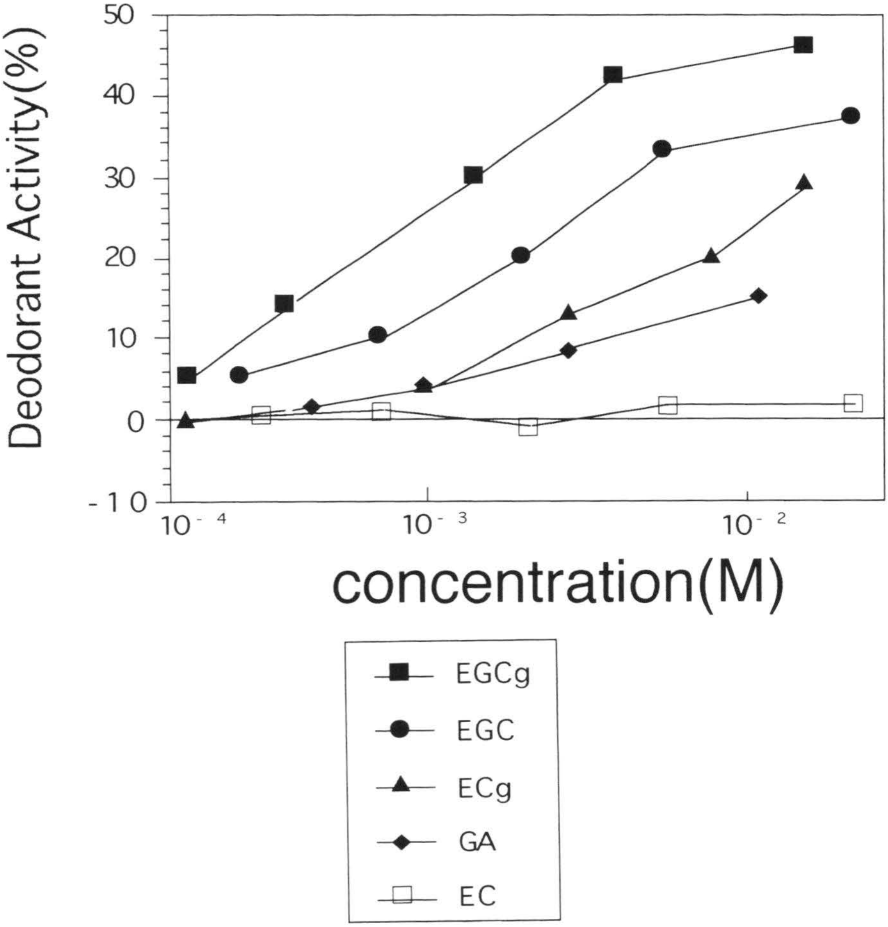


Fig. 1 : Comparison of the deodorizing activity (%) against CH_3SH of tea catechins and gallic acid. For significance of abbreviations see the previous page.

Materials and Methods

This study used many analytical instruments. ^1H- and ^{13}C-NMR spectra were recorded with a Bruker AM 400 spectrophotometer at 400 Mhz and SIMS data were measured with a Hitachi M 80 A instrument. Preparative high performance liquid chromatography (HPLC) was performed by a Shimadzu LC-8A system with a Senshu-pack ODS-5251-SH column monitored at 280 nm. The deodorizing activity of each sample against CH_3SH was assayed by gas chromatographic analysis of the headspace gas, a Shimadzu GC-9A instrument equipped with a 5mx3mm i.d. glass column packed with 20% DOP

on Chromosorb W AW-DMCS 60-80 mesh being used. CH_3SH was detected by a flame photometric detector (FPD). The analytical methods are illustrated in Fig. 2, 3 and 4 and Table 2.

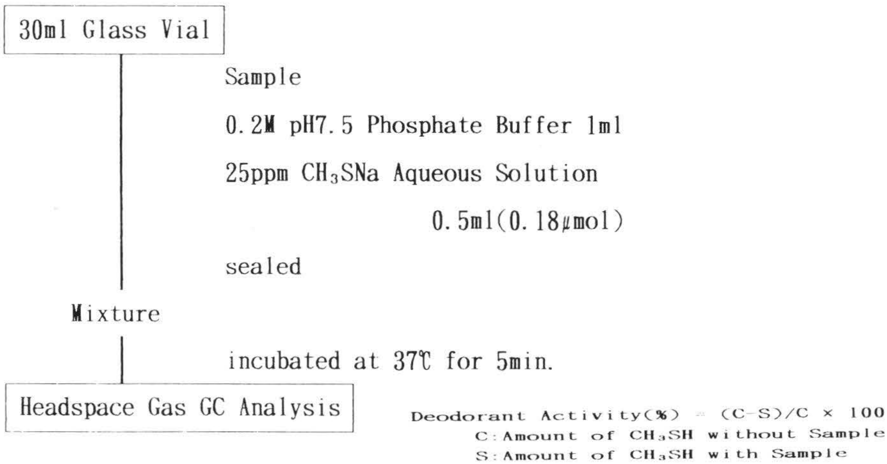


Fig. 2 : Deodorizing assay by GC analysis.

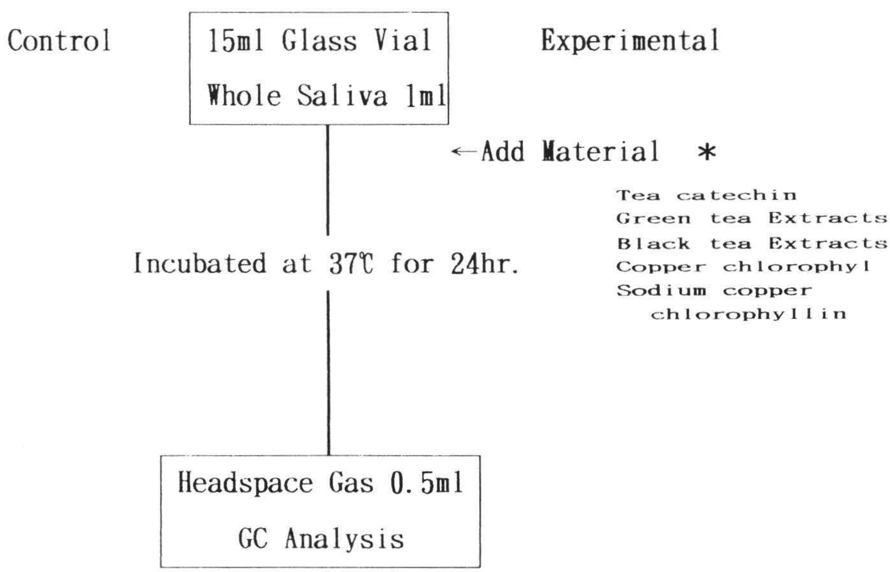


Fig. 3 : Comparison of deodorizing materials

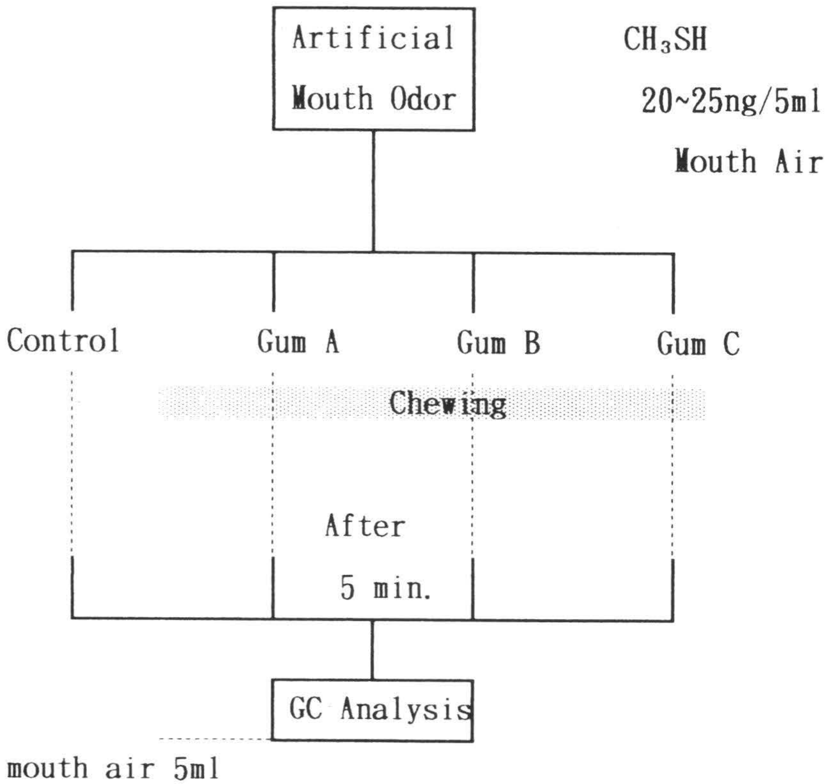


Fig. 4 : Effect of chewing gum containing tea extract.

Table 2 : Manufacturing of chewing gum

	Gum A	Gum B	Gum C
Green tea extract	-	50 ppm	-
Copper chlorophyl	-	50 ppm	50 ppm
Tea catechin	-	-	250 ppm
Black tea extract	-	-	50 ppm

Results

Figure 1 shows the deodorizing effect of each component of tea catechins such as EGCg, (-)-epicatechin gallate (ECg), (-)-epicatechin (EC), (-)-epigallocatechin (EGC) and of gallic acid (GA) against CH_3SH. This assay

was performed in a potassium phosphate buffer solution (pH 7.5) at 37°C for 5 min consistent with oral use. The gallocatechins, EGCg and EGC, which have three hydroxyl groups at the 3', 4' and 5' positions of the B-ring, showed high deodorizing activity, the activity order of these tea catechins being EGCg > EGC > Ecg > GA > EC.

To better understand the deodorizing mechanism of tea catechin, we have limited our present study to the deodorizing reaction of EGCg, and we tried to find whether EGCg would chemically react with CH_3SH to give new products. The reaction mixture was analyzed by HPLC. The peak of EGCg decreased with increasing time, and new peaks appeared. Respectively, these are named peak I, peak II and peak III (Fig. 5).

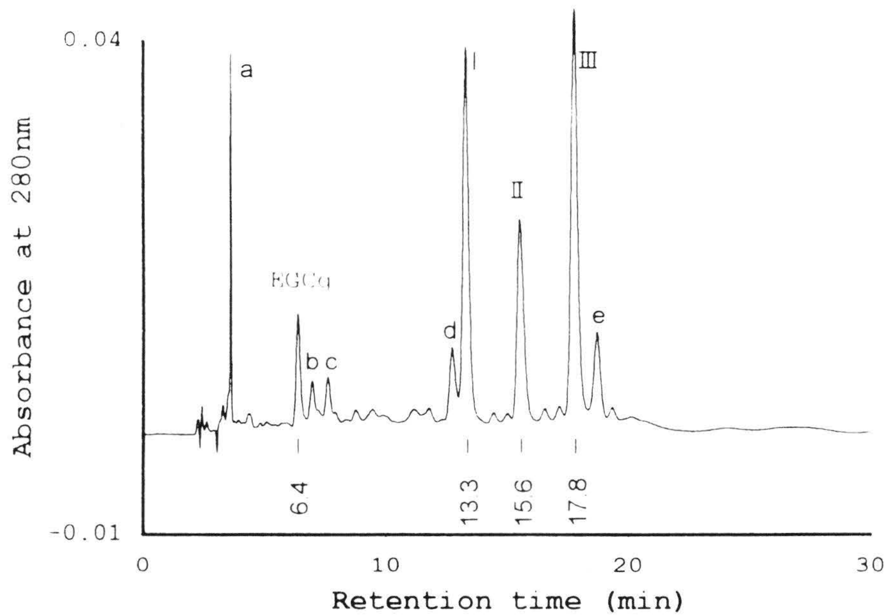


Fig. 5 : HPLC chromatogram at 280 nm of the reaction mixture between EGCg and CH_3SNa at 45 min.

Compound 1 contained one proton at the 6' position of the B ring and a methyl group as revealed by the ^1H-NMR spectrum (δ 6.75 and 2.27). SIMS and elemental analysis of compound 1 gave an ion of m/z 505 (M+H) and the formula C13H20O11S.2H20 (found C, 51.00%; H, 4.48%; S, 5.92%; calculated: C, 51.11%; H, 4.48%; S, 5.93%). From these analytical data,

compound 1 was identified to be 2'-methylthio EGCg. The ^{1}H-NMR spectrum of compound 2 was similar to that of compound 1, except for the absence of a 6' proton signal and the appearance of a six proton siglet signal (δ 2.28) due to two methylthio groups. SIMS data for compound 2 gave an ion of m/z 551 (M+H) corresponding to the structure of two methylthio groups being introduced to EGCg. The ^{1}H-NMR spectrum of compound 3 was almost the same as that of compound 2, but it exhibited a methylthio group signal and another downfield-shifted methyl signal (δ 2.35 and 3.22). SIMS data for compound 3 gave an ion of m/z 567 (M+H). Based on the SIMS and NMR data, compound 2 and 3 were idientified as 2', 6'-dimethylthio EGCg and 2'-methyl-sulfinyl-6'-methylthio EGCg, respectively. Compounds 1, 2 and 3 have not been previously reported. The compound constituting peak II was assumed to be 2', 6'-dimethylthio gallocatechin gallate, which is epimerized at the 3 position of compound 2. The isolation of peak II in a quantity sufficient to perform NMR, mass spectrometry and a deodorizing assay are underway in more detail.

The effect of atmosphere oxygen in our deodorizing assay system was examined (Fig. 6). Under the ordinary conditions of the CH_3SH deodorizing assay, the amount of EGCg decreased over time, and compound 1 appeared in the mixture. However under anaerobic conditions with helium gas, the

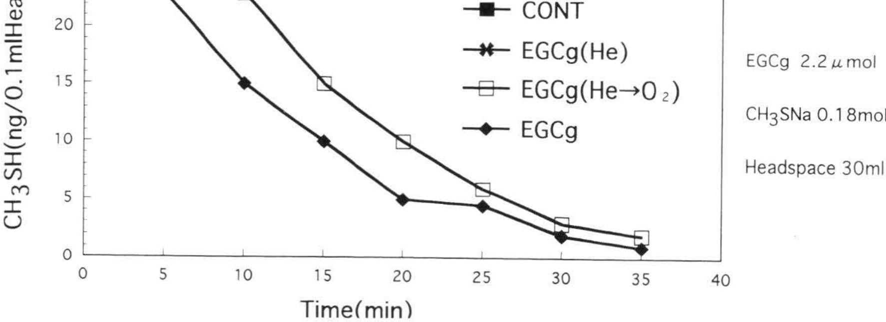


Fig. 6 : Effect of O_2 and helium gas on the CH_3SH deodorizing action of EGCg.

amount of EGCg did not decrease and EGCg showed no deodorizing efficiency. Under these conditions, the injection of O_2 into the sealed tube at 6min subsequently restored the deodorizing activity of EGCg. In the absence of O_2, EGCg remained intact, and no peak for compound 1 was observed in the mixture. We assume the possible reaction pathway between EGCg and CH_3SH as follows (Fig. 7). Tri-phenolic hydroxyl groups in the B ring of EGCg were oxidized with atmosphere oxygen to ortho-quinone as the intermediate.

Compounds 1, 2 and 3 are considered to have been intermediates in the reaction, and the deodorizing ability of each of these compounds was determined. The results are summarized in Table 3. The deodorizing activity of each of compounds produced in the reaction was stronger than that of EGCg as the starting material and of sodium copper chlorophyllin, which is commercially available as an oral deodorizer. We assume that the introduction of a methyl-sulfinyl group at the 2' position resulted in a lower electron density in the B ring and activation of the 6' position. It is interesting that compounds 2 and 3, which were already substituted at the 2' and 6' positions, had moderate activity. It is considered that CH_3S might be more easily introduced into the B ring than into the galloyl group in EGCg. A third CH_3S may be introduced into the galloyl group of compounds 2 and 3.

Table 3 : Deodorizing activity (%) of EGCg and other compounds

Compound	Amount (mg)	DA (%)
Sodium copper chlorophyllin	5	28
Sodium copper chlorophyllin	1	6
EGCg	1	28
2'-methylthio EGCg (Peak I)	1	56
2', 6'-Dimethylthio EGCg (Peak II)	1	35
2'-methylsulfinyl-6'-methylthio EGCg (Peak III)	1	43

DA : deodorizing activity

Conclusions

EGCg showed deodorizing activity against CH_3SH by a chemical reaction between EGCg and CH_3SH. The non-volatile reaction product were identified to be compounds introducing a methylthio and/or a methylsulfinyl group into the B ring of EGCg, and gaseous oxygen was necessary for deodorizing activity. The deodorizing mechanism of EGCg was due to the addition of the methylthio group to the ortho-quinone generated by

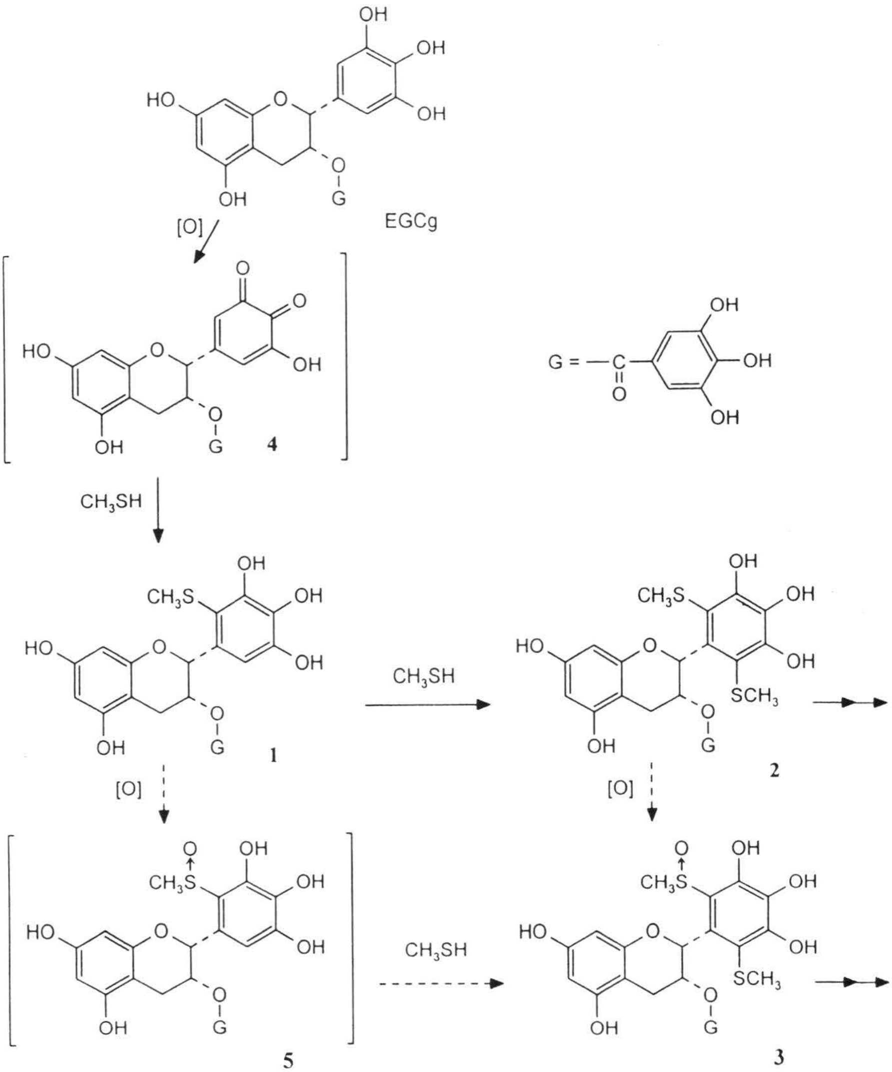


Fig. 7 : Reaction products between EGCg and CH_3SH and the possible production pathway.

atmosphere oxygen. Secondary compounds produced by the reaction between EGCg and CH_3SH had a stronger deodorizing activity than that of EGCg itself.

The data suggest that chewing gum containing tea extracts has deodorizing activity for halitosis. These results suggest that chewing of gum containing tea extracts is useful to decrease bad breath.

Consensus Reports

Consensus Report 1 :
The Clinical Approach of Breath Malodour

Chairman : M. Rosenberg
Secretary : L. Feenstra and J.M. Coil

Definition

Breath malodour is a recognisable condition which deserves professional attention. It should receive recognition of the professional community, in the medical/dental curriculum and in the public at large.
Clinical evaluation of breath malodour should include organoleptic comparison of the odour coming from the nose and mouth, preferably by more than one examinator. Diagnostic criteria, validation, testing and assessment procedures should be developed and existing ones compared.
Breath malodour is a condition which may have a variety of etiologic origins and different treatment approaches, which should be clearly stated to the public and patients. Financial relationships of clinicians to manufacturers of endorsed products should be disclosed.
Patients should be encouraged to bring a "confidant" (e.g. spouse) to consultations and to ask family members to help monitor the problem.
Breath malodour patients should be advised to strive towards optimal oral hygiene including brushing of the back parts of the tongue.
Future research should also be directed towards a better understanding of the psychological aspects of breath malodour.

Consensus Report 2 :
Toothpastes and Mouthwashes to Control Breath Malodour

Chairman : M. Newman
Secretary : S. Raven

Scope of the group

The group principally focused on the toothpastes and mouthwashes. However it was agreed that other product forms such as freshener pills and oral irrigation devices could be considered in the future.

The group discussed

"The need to standardize problems in order to establish quantitative guidelines for product efficacy".

The principle need is to define whether the product works and delivers a reduction in breath malodour. The problem of breath malodour can be defined as either a short-term, transient problem or a long-term chronic problem. The group agreed that commercial products address the short-term problem which includes conditions such as morning breath or food odours.

The duration that the product should be effective was discussed and a consensus view reached that the product should be effective for at least 3 hours.

Type of benefit

No consensus was reached on the type of benefit with the group split between self perceived benefit or a third party assessment. Both benefits should be considered.

Magnitude of benefit

Without standard protocols it is not possible to define what constitutes an effective product in terms of odour reduction. It was agreed that the product

should be supported by an appropriate clinical trial against relevant controls. The definition of a suitable control was debated and the final view was that there is no obvious negative or positive "Gold standards". It was agreed that studies should contain a placebo treatment preferably identical to the test product except for active ingredients. The exception may be for the new treatment regimens. A two level system was proposed for mouthwashes and toothpaste given the general belief that mouthrinse has a greater potential to deliver efficacy.

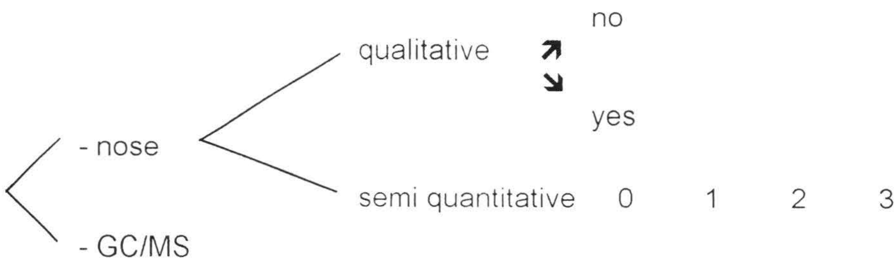
Future objectives

The future of the workgroup should focus on further definition of the standardized procedures. The ultimate aim would be to allow practitioners/consumers to make an informed choice based on proven product efficacy.

Consensus Report 3 :
Value of Commercially Available Breath Malodour Meters

Chairman : I. Kleinberg
Secretary : A. Spielman

1. What is the gold standard to measure bad breath?



2. Which volatile compounds are present in the oral cavity as part of bad breath?
a) volatile sulphur compounds
b) volatile organic acids
c) indole and skatole
d) amines

3. How do we measure these volatile compounds?
a) gas chromatography/mass spectrometry with a sulphide monitor
b) portable volatile sulphur compounds monitor
c) other diagnostic methods

4. Problems with the currently available diagnostic methods?
   Portable Sulphide Monitors
- are semiquantitative monitoring tool
- are good for hydrogen sulphide
- are not so good for methyl mercaptane
- are not good for the rest of VSC
- correlation factor between organoleptic measurements and Halimeter™ (Interscan Corp., Chatsworth, CA, USA) is 0.58 (based on published data)
- problems with calibration and standardization of the instrument

5. Recommendations

a) development of more specific diagnostic methods
b) the ideal method should be simple, and should detect all four classes of volatile compounds of the bad breath cocktail
c) the diagnostic methods should have influence on the choice of treatment
d) we need new devices
   - for better diagnosis
   - to monitor treatment choice
   - to monitor treatment progression
e) more basic research is needed
f) a task force is needed to recommend diagnostic method development

Drukkerij en Binderij
SCHEERDERS van KERCHOVE
9100 ST.-NIKLAAS

Leuven
University
Press